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TITLE: Eosinophil Cell Lines in a Tri-Cell Multicellular Tumor spheroid (MTS)/ Endothelium Complex: Down Regulation of Adhesion & Integrin Molecules-Implications of Metastasis Inhibition

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14. ABSTRACT

Histopathological examination of tumor specimens have revealed eosinophil infiltrates and spread of granular contents. The data, however, remain equivocal as to the prognostic significance of tumor associated eosinophils. Eosinophils play a key role in inflammatory allergic asthma and as anti-helminthic agents. We hypothesized that eosinophils could also exert toxicity on tumors and because they bind to and infiltrate tumors, that this effect occurs from within. Moreover this effect is due to released mediators that include cytokines such as IL-4, IL-10 that are preformed and which have anticancer and immunomodulatory properties. Initial studies in our laboratory have shown that activated eosinophils: 1) inhibit the in vitro growth of breast tumor cells: 2) bind to and infiltrate tumor spheroids and that this action is increased greatly by IL-5. When cultured with HUV-EC-C monolayers, the MTS with and without eosinophils attach to the HUV-EC-Cs and over time (24hrs) spread from the spheroid onto the HUV-EC-C lawn. Recent data demonstrate that eosinophil-infiltrated tumor spheroids become loosely aggregated and individual cells are killed. These studies demonstrated the potential of this Tri-Cell Model System to further delineate the influence of eosinophils on tumor attachment to the extracellular matrix.

15. SUBJECT TERMS
MCF-7 multicellular tumor spheroid (MTS)

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Introduction

Eosinophils are nonimmune inflammatory cells which are intricately involved in helminthic infections and allergic hypersensitivity reactions (1, 2). Activated eosinophils produce a host of mediators, including cytokines which can be both detrimental as well as helpful to the host (3, 4, 5). Cytokines such as IL-1α, IL-1β and TNFα, also produced by eosinophils, upregulate adhesion molecule expression on both endothelial and tumor cells (6, 7). Increased expression of adhesion molecules such as ICAM-1, V-CAM1 and E-Selectin (ELAM-1) on tumor cells is correlated with increased invasiveness and metastasis (8, 9, 10).

Multicellular tumor spheroids are three-dimensional cell culture systems which have been shown to model the growth characteristics of tumors as well as their metastatic potential(11, 12, 13). In light of this, tumor spheroids have more recently been used as a model for examining tumor-endothelial cell interactions in order to better understand the interactions of the tumor with its microvascular environment.

We have utilized the tumor spheroid model system to study not only its interaction with endothelial cells, but also examine the effector role of eosinophils in tumor:endothelial cell interaction. We have shown that peripheral blood eosinophils from allergic and asthmatic individuals can inhibit the growth of both breast and prostate tumor cells (14, 15). In this study we attempted to examine the hypothesis that eosinophils infiltrate breast spheroids (14) release of their contents into the tumor microenvironment and that these eosinophil mediators, to include cytokines, can modulate spheroid adherence and invasion. In order to do this we attempted to set up an eosinophil tumor spheroid:endothelial tri-cell complex (16).

Body

Collection of Conditioned Supernatants from Peripheral Blood Eosinophils from allergic Individuals and from IL-5 treated Eosinophils Cell Lines. Peripheral blood eosinophils from moderate to severely allergic individuals were incubated at 1 x 10^6/ml for 24hrs in RPMI medium supplemented with penicillin/streptomycin (100 units/ml and 100ug/ml, respectively), gentamycin (50 ug/ml) and 1% fetal bovine serum. The supernatants were collected after centrifugation at 2,000 rpm for 30 min, aliquoted and stored at -80C. Eosinophil cell lines (1 x 10^6/ml) were pulsed with IL-5 (5ng/ml) then cultured for 24hrs; supernatants were collected and stored at -80C.

Eosinophil Cell Line Conditioned Supernatants. The advantage this work has over that of most studies with eosinophils is that we were able to utilize cell lines which have been established in our laboratory. We utilized hypo- and hyperdense eosinophil cell lines: GRC.014.22, GRC.014.24, sublines of GRC.014.24; BJA.060.22, BJA.060.24; SD.031.22 and SD.031.24. All cell lines were pulsed with IL-5 (5ng/ml), then cultured for 24hrs, 48hrs and 72hrs. The conditioned supernatants were collected and stored at -80C.

Collection of Conditioned Supernatants from MRC Fibroblasts. MRC fibroblasts were

seeded into T_{25} tissue culture flasks and allowed to grow to confluency (2-3 days), after which the medium was collected and centrifuged at 2,000 rpm for 30min. Supernatants were collected, aliquoted and stored at -80C.

Cytokine Gene Expression. Total RNA from eosinophil cell lines was extracted using purescript RNA isolation kits from Gentra Systems. RNA integrity was assessed with agarose gel electrophoresis and ethidium bromide staining. Extracted RNA was quantified by spectrophotometry.

Real Time Assays

Relative quantitation of gene expression was conducted with the ABI Prism 7700 Sequence Detection System using 96-well cytokine predeveloped assay kits from Applied Biosystems. Briefly, 1μg of total RNA was reverse-transcribed using Taqman Transcription Reagents (Applied Biosystems). This is the first step of the two-step RT-PCR reaction. The second step is a RT- PCR step using Taq Man Universal PCR Master Mix. Gene expression was quantitated relative to the GAPDH housekeeping gene control. The threshold cycle or CT value was used in this quantitation.

Generation of Multicellular Tumor Spheroids. Subconfluent monolayer cultures of MCF-7 and MDA-MB-231 nonmetastatic and metastatic tumor cells respectively, were maintained in RPMI medium supplemented with penicillin/streptomycin and gentamycin, and 10% FBS (complete medium) at 37C, 95% air, 5% CO_2. After trypsinization and cell count, the cells were dispensed into T_{25} non-vented culture flasks (1 x 10^6 cells/flask with 10mls RPMI) and rocked in a 37C incubator, agitating at 30 periods per minute for 24-48hr. The spheroids were then transferred to 100 mg culture dishes containing an overlay of 0.5% noble agar in 10% RPMI medium. The dishes then incubated at 37C in a 5% CO_2 atmosphere for 7 days with regular feeding. This was our initial method of generating MCF-7 spheroids. With this method we were able to generate approximately 200 spheroids per flask. Spheroids ranged in size from 200um to 1200um with an average size of 800um. In the past year we had to switch methods of generating spheroids because of equipment problems. We used a modified method of Yuhas *et al* (17). Briefly tumor cells were dispensed into 96-well plates containing an agar overlay (0.5% noble agar) at $3x10^3$ cells per well. The plates were incubated at 37C, 5% CO_2, 95% air for 24 to 48hrs. The spheroids were transferred similarly to 6-well cluster plates with an agar overlay. The major differences in the spheroids formed by this method included size and clonal development. Spheroids which developed from single cells, ranged in size from 100um to 400um, with the average size being 200um.

Eosinophil Cell Lines. Hypo and hyperdense eosinophil cell lines were maintained in RPMI complete medium, subculturing every 2 to 3 days in T_{75} tissue culture flasks at 37C, 5% CO_2 95% air atmospheric conditions.

Endothelial Cells. Two endothelial cell lines, human umbilical vein endothelial cells HUV-EC-C and Human abdominal aorta endothelial cells (HAEE-1) were maintained in RPMI complete

and F-12K complete medium, respectively. Both media were supplemented with endothelial cell growth supplement (ECGS). For experimental purposes either HUV-EC-Cs or HAEE cells were seeded into 6-well cluster plates at 3 x 10^5 cells/well and incubated overnight at 37C, 5% CO_2, 95% air. Some experiments were set up using 8 chamber tissue culture slides. In this case, HUV-EC-Cs were seeded at 0.5 x 10^5 cells per chamber

Eosinophil:MTS:Endothelial Tri-Cell Complex. MDA-MB-231 tumor cells and HC-202 tumor cells failed to form spheroids. 7-day old MCF-7 MTS were cultured overnight with medium alone, hypodense eosinophils pretreated with IL-5 at 5ng/ml or with IL-5 present in the culture medium. MTS with associated eosinophils were then added to the endothelial monolayers and further cultured for 1, 4 and 16-24hrs.

Each treatment was set up in duplicate wells. The medium was removed, the wells/chambers washed with PBS then stained with May Grunwald Giemsa. For the 4hr and 16-24hr cultures, one well of each duplicate was stained with FITC -labelled anti-eosinophil peroxidase antibody. The slides and culture wells were examined microscopically and documented by photomicrographs. Eosinophils and MCF-7 cells (suspension) were cultured with HUV-EC-C monolayers for 24hrs and the complexes were stained with triple stains (FITC- Eosinophils, Texas Red- HUV-EC-Cs and DAPI- MCF-7).

We experienced tremendous difficulty with the triple fluorescence stains in that the integrity of the secondary antibody appeared to be lost i.e. eosinophils which were stained for FITC were staining blue for DAPI and vice versa. This made interpretation difficult. We then used the vital stain PKH26 and pre-stained eosinophils before co-culturing with the MCF-7 MTS. PKH26-stained eosinophils were co-cultured with 7-day old MCF-7MTS for 3 and 24hr, after which results were collected by photo documentation of video graphs.

MRC-5 supernatants were negative for IL-1, IL-4, IL-5, TNFα, IL-10 and IL-12 and were not used in the study.

Invasion Assay. BD BioCoat Matrigel invasion chambers were used to evaluate the influence of eosinophils on tumor cell invasion. Because the metastatic cell line, MDA-MB-231 failed to form spheroids, we utilized single cell combinations of eosinophils and tumor cells. The Matrigel Invasion Chamber consists of a 24-well tissue culture plate with cell culture inserts containing 8 micron pore size PET membranes with a thin layer of matrigel basement membrane matrix. The method used was that described by the manufacturer. Briefly, cultured cells were prepared at 5x10^4 cells/ml. A chemoattractant (in our case RPMI containing 10% fetal bovine serum) waas added to the wells of the plate (wells minus the membrane inserts). The cells (0.5 ml) were added to the chamber containing the membrane/matrigel and this was then placed into the well containing the chemoattractant. The plates were incubated for 22 hr in a humidified incubator at 37C, 5% CO2 atmosphere. After incubation, the noninvading cells were removed from the upper surface of the membrane by gently scrubbing with a cotton-tipped swab (2X).

The cells on the lower surface (invaded cells) were fixed and stained. In the experiments described here, the membranes were gently cut with a scapel and gently teased away with tweezers and placed botton side down on a slide. Phase-contrast photomicrographs were obtained. In the case of complexing 2 or more cell types, first a lawn of cell #1 (endothelial or tumor) was allowed to form, then cells #2 and #3 were added (if 2 cells, first one then the other).

Flow Cytometry. The effect of cytokines on cell adhesion molecules on MCF-7 and MDA-MB-231 tumor cells was determined by flow cytometry. Subconfluent (50-70%) T_{75} flasks of MCF-7 and MDA-MB-231 tumor cells were cultured for 24 hr in medium containing IL-1 (5ng/ml), IL-4 (10ng/ml) or combination of the two, after which the cells were harvested, counted and prepared for flow cytometry analysis. ICAM-1 expression was detected by **a.direct immunofluorescence staining**. Briefly, the cells were adjusted to $2x10^7$ cells/ml, 50 ul of which was mixed with 50 ul of PE-labelled anti-CD54 antibody. The mixture was then incubated on ice for 30-45 minutes. The cells were then washed and resuspended in 0.5 ml cold phosphate buffered saline and maintained on ice until analyzed. Prior to analysis the cells were fixed with paraformaldehyde. VCAM-1 and ELAM-1 surface molecules were assessed by **b.indirect immunofluorescence staining.** As previously described, cytokine-treated cells were harvested and counted, adjusting counts to $2x10^7$ cells/ml. Primary antibody (anti-VCAM-1 or anti-ELAM-1) was incubated with the tumor cells on ice for 30 -45 min. The cells were then washed then incubated with fluorescent-labelled secondary antibody. The cells were then prepared as described above for flow cytomentry analysis.

Figure 1. *Cytospin Preparations of Eosinophil Cell Line.* Eosinophil cell-lines stained with hematoxylin and eosin (A) giemsa maygrunwald (B) and luxol fast blue (C). Vacuoles and granules can be readily seen.

Figure 2. *Eosinophil 24hr Supernatants.* Of the six donors tested, only donors 5 and 6 (both hypo-and hyperdense eosinophils) contained supernatants that were positive for IL-4. Conditioned supernatants from hypodense eosinophils from donors 1, 2 and 4 were positive for IL-5 while hyperdense eosinophils from only donors 1 and 4 were positive. Neither of the six donor eosinophils produced IL-12.

Figure 3. *Eosinophil cell lines produce IL-5 I.* 24hr supernatants from hypodense eosinophil cell line BJA.060 contained comparable amounts of IL-5 as did the peripheral blood eosinophils, ≥200pg/ml while 72hr conditioned supernatants from hyerdense eosinophils produced 50% less IL-5.

Figure 4. *Presence of IL-5 in cultured supernatants of eosinophil cell lines II.* Cultured supernatants from the allergy/asthma positive, breast cancer positive hypo- and hyperdense eosinophil cell line cultured supernatants were more potent for IL-5, producing >200pg/ml IL-5 at 24hr, 48hr and 72hr (hypodense) and 24hr and 72hr (hyperdense).

Figure 5. *Eosinophil sublines produce IL-5 III.* The $CCR3^+$ (eotaxin receptor positive) subline, GRC.014.24S1 was the most effective of the three lines in producing IL-5 in comparable amounts to that of the peripheral blood eosinophils, ≥300pg/ml. When this line was sorted for the VLA-4 marker, (GRC.014.24S1.CD49D) the amount of IL-5 dropped precipitously to that

of the parent line (24hr and 48hr).

Figure 6. *Baseline cytokine gene expression in eosinophil cell lines I.* Both cell lines, GRC.014.22 (A) and GRC.014.24 (B) cell lines expressed the array of cytokines shown, at varying levels. The hypodense cell line (A) expressed slightly higher levels than the hyperdense cell line (B).

Figure 7. *Baseline cytokine gene expression in eosinophil cell lines II.* Similar results were observed for cell lines BJA.060.22 (A) and BJA.060.24 (B) as was observed in figure 6, i.e., the hypodense cell line BJA.060.22 (A) produced slightly higher amounts of IL-5 thatn the hyperdense line, BJA.060.24 (B).

Figure 8. *MCF-7 Multicellular Tumor Spheroids.* MCF-7 multicellular tumor spheroids after three (A, C) and 7 (B, D) days in culture at 37C on 0.5% noble agar containing RPMI complete medium.

Figure 9. *Effect of Interleukin-5 on MCF-7 spheroid growth.* IL-5 altered the growth of the MCF-7 spheroid in a dose-dependent fashion. The integrity of the spheroid was compromised with the lowest concentration of 0.5ng/ml (B) and necrosis at the core was observed with 5 and 10ng/ml. (E and F, respectively)

Figure 10. *Effect of Interleukin-4 on MCF-7 multicellular tumor spheroid growth.* IL-4 similarly altered the growth and integrity of the MCF-7 spheroid. In figure 10B the spheroids attached and began to spread. Necrosis at the core was observed with concentrations as low as 1ng/ml.

Figure 11. *MCF-7 Multicellular Tumor Spheroids Bind to HUV-EC-C Monolayer.* MCF-7 multicellular tumor spheroids cultured on human umbilical vein endothelial cell (HUV-EC-C) monolayer after 1hr (A) 24hr (B) and 48hr (C) culture. Some spheroids developed necrotic cores overtime (B). As the spheroid binds tightly to the HUV-EC-Cs, cells began to grow out from the spheroid forming a monolayer over the HUV-EC-Cs (B, C) often destroying them overtime.

Figure 12. *Eosinophil:MCF-7 MTS Co-culture on HUV-EC-C* Monolayers. Eosinophils were first cultured with MCF-7 multicellular tumor spheroids for 24hrs then cultured on a lawn of HUV-EC-C cells. The solid arrows point to esoinophils bound tightly to the spheroids (A, B) as well as unbound eosinophil clusters (C). In figure 12C, the eosinophils completely surround the spheroid. Figure 12D shows high power resolution of a MCF-7 MTS with bound eosinophils attached to HUV-EC-C cells. The hatched arrow points to an endothelial cell.

Figure 13. *Histological Examination of Eosinophil:MCF-7 MTS on HUV-EC-C Monolayer (1hr co-culture).* Eosinophils were cultured with MCF-7 MTS for 24hrs, then placed on a lawn of HUV-EC-Cs for 1 hr in a Falcon 8-chamber tissue culture slide. The slide was then washed with

PBS, fixed with methanol, then stained with giemsa maygrunwald for the presence of eosinophils. Figure 13A shows a spheroid attached to HUV-EC-Cs. 24hr co-culture of eosinophils with MCF-7 spheroids resulted in the eosinophils binding and infiltrating the spheroid. In 13B the arrow is pointing to an attached eosinophil which can more readily be seen at 100X in 13C. Figure 13D is from a duplicate well and the arrow is pointing to eosinophil granules which are easily seen at the higher power (13E). Eosinophil granules are seen throughout the spheroids in 13C and 13D.

Figure 14. *Histological Examination of 24hr IL-5 Treated Eosinophil:MCF-7 MTS on HUV-EC-C Monolayer (1hr co-culture).* Eosinophil:MTS cultures were treated with interleukin-5 (IL-5) at 5ng/ml (24hr), then cultured on HUV-EC-Cs for 1hr. As was the case with the non-IL-5 treated Eosinophil:MTS co-cultures, eosinophils can be seen attached to both the spheroids and the HUV-EC-C.

Figure 15. *Histological Examination of MCF-7 MTS and 24hr IL-5 Treated Eosinophils on HUV-EC-C Monolayer (1hr co-culture).* When eosinophils were first incubated with IL-5 (24hr), then cultured with MTS and HUV-EC-Cs for 1hr, the eosinophils were more activated and bound more readily to the HUV-EC-Cs, preventing the tumor spheroid from binding.

Figure 16. *Histological Examination of Eosinophil:MCF-7 MTS on HUV-EC-C Monolayer (24hr co-culture).* MCF-7 spheroids post 24hr culture with eosinophils began to spread out along the HUV-EC-C monolayer (fig. 16A). The presence of eosinophils on and within the spheroid does not affect the spreading of the tumor cells, however the eosinophils remain bound to the tumor cells as they spread out (fig 16B, 16C, 16D). Eosinophils can be seen at the arrow heads and more clearly at 40X (fig. 16E) and 100X (fig. 16F) magnification, respectively. The hatched arrow is pointing to a tumor cell that has moved out from the spheroid with eosinophils attached (16E). 24hr cultures reveal more movement of tumor cells out from the spheroid and attachment of eosinophils (16B, 16C). Figure 16D and 16E are magnifications of 16B and 16C showing tremendous eosinophilic presence (arrows).

Figure 17. *Histological Examination of 24hr IL-5 Treated Eosinophil:MCF-7 MTS on HUV-EC-C Monolayer (24hr co-culture).* As was the case with the 1hr cultures, eosinophil-infiltrated MCF-spheroids were pretreated with IL-5 (5ng/ml) then seeded onto HUV-EC-C monolayers which were further cultured for an additional 24hrs. Prior to co-culture, eosinophils were pretreated with IL-5 as described previously.

Figure 18. *Histological Examination of MCF-7 MTS and 24hr IL–5 Treated Eosinophils on HUV-EC-C Monolayer (24hr co-culture).* These data again show massive involvement of eosinophils with MCF-7 tumor spheroids and also with endothelial cells. IL-5 pretreatment of eosinophils induced greater activation of the eosinophils than the other two treatments (IL-5 treated eosinophils spheroids; IL-5 present in the culture medium with eosinophils and spheroids).

Figure 19. *Photomicrograph of Stained Section of Eosinophil:MCF-7 MTS (19A) and Electron Micrograph of Fine Section of Eosinophil:MCF-7 MTS (19B).* Eosinophil-infiltrated spheroid

was sectioned and stained with hematoxylin and eosin (fig. 19A). The core of the spheroid contained many esoinophils which can be seen throughout the sectioned spheroid. Moreover transmission electron micrograph of a thin section of the spheroid was also prepared and again granulated esoinophils can readily be seen throughout the section (arrows, figure 19B).

Figure 20. *Photomicrograph of fluorescent staining of eosinophils, HUV-EC-Cs and MCF-7 cells.* Cells were stained individually with fluorescent dyes FITC, Texas Red and DAPI, respectively. The marker antigens used were Major Basic Protein (Eosinophils), von Willibrand Factor (HUV-EC-C) and Epithelial Cell Antigen (MCF-7).

Figure 21. *Photomicrograph of dual cell complexes of eosinophil:HUV-EC-C, MCF-7:HUV-EC-C.* In figure 21A the arrow points to an eosinophil binding to HUV-EC-C cells and in figure 21B MCF-7 tumor cells binding to HUV-EC-C.

Figure 22. *Triple staining of eosinophil:MCF-7:HUVEC tri-cell complex.* MCF-7 tumor cells ($1x10^3$) were added to HUV-EC-C monolayers and incubated at 37C after which hypodense eosinophils were added at 100:1 Eosinophils to tumor ratio. The plates were further incubated for 24hrs. The cultures were then stained with triple stain FITC, DAPI and Texas Red. The solid arrow points to an eosinophil and the hatched arrow points to a tumor cell, while the red cells are HUV-EC-Cs.

Figure 23. *Photomicrographs of PKH26-Stained Eosinophils Co-Cultured with MCF-7 MTS.* 7-day old MCF-7 MTS were cultured with hypodense eosinophils at 100:1 E.T ratio (200um MTS contains approximately $1x10^5$ cells) for 3 and 24hrs at 37C. The eosinophils had been prestained with the fluorescent vital stain PKH26. Figures A and C are phase contrast photomicrographs of eosinophils binding to or bound to the spheroid and in C the spheroid appears to be disintegrating. In figures B and D, respectively, eosinophils can be seen bound (3hrs) and completely infiltrating the spheroid (24hr) which has lost its integrity.

Figure 24. *Invasion of HUV-EC-C, MCF-7 and MDA-MB-231 tumor cells in the absence of chemoattractant.* Little to no tumor cells were found in the absence of chemoattractant.

Figure 25. *Invasion of tumor cells in the presence of eosinophils.* Within 24hr, both HUVEC cells and >90% of the eosinophils were found on the under surface of the membrane, while very few of the MCF-7 tumor cells had invaded the matrix, but >50% of the eosinophils were found on the underside of the matrix membrane complex. In figure 25C, >50% of the MDA-MB-231 tumor cells, but <50% of the eosinophils had invaded the matrix.

Figure 26. *Invasion of tumor cells from eosinophil:tumor:HUVEC tricell complex.* Unlike in figure 25C, far fewer MDA-MB-231 tumor cells were found invading the matrix. Also there were far fewer eosinophils present.

Figure 27. *The effect of IL-1 and Il-4 on ICAM-1 expression on MCF-7 tumor cells.* MCF-7 tumor cells were negative for ICAM-1 (A). Both IL-1 and IL-4 induced ICAM-1 expression (B and C, respectively), IL-4 greater than IL-1. The combination treatment of IL-1 and IL-4 induced ICAM-1 expression over that of either cytokine alone (D).

Figure 28. *The effect of IL-1 and IL-4 on VCAM-1 expression on MCF-7 tumor cells.* VCAM-1 was present on MCF-7 tumor cells. Treatment with IL-1 and IL-4, alone and in combination had no effect on its expression.

Figure 29. *The effect of IL-1 and IL-4 on ICAM-1 expression on MDA-MB-231 tumor cells.* High levels of ICAM-1 were expressed on the surface of the metastatic tumor cell line, MDA-MB-231. Neither IL-1 nor IL-4 was able to further increase expression. However, the IL-1/IL-4 combination caused a slight decrease in expression.

Figure 30. *The effect of IL-1 and IL-4 on VCAM-1 expression on MDA-MB-231 tumor cells.* VCAM-1 was also presenet on MDA-MB-231 tumor cells. IL-1 and IL_4 had no effect (B and D, respectively). When IL-1 and IL-4 were added simultaneously, there was a slight decrease in expression (D) similarly to that seen with ICAM-1 in figure 29 D.

Figure 31. *The effect of IL-1 and IL-4 on ELAM-1 expression on MDA-MB-231 tumor cells.* Moderate levels of ELAM-1 were found on MDA-MB-231. No further expression was observed post treatment with IL-1, IL-4 and the two cytokines together.

Key Research Accomplishments

Promotion to Associate Professor

Invited to be "Guest Editor" for Cellular and Molecular Biology

Invited to be Main Speaker at the 7th International Conference of Anti-cancer Research, Corfu, Greece.

Invited to contribute an original or review article of a special issue of *in vivo* on "New Anti-cancer Agents: *In Vitro* and *In Vivo* Evaluation".

Reportable Outcomes

Furbert-Harris PM, Laniyan I, Harris D, Dunston GM, Vaughn T, Abdelnaby A, Parish-Gause D, Oredipe OA. Activated eosinophils infiltrate MCF-7 breast multicellular tumor spheroids. Anti-cancer Res. 2003 Jan-Feb; 23(1A):71-8.

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Furbert-Harris, P.M., Parish-Gause, D., Laniyan, I., Howland, C., Abdelnaby, A., Vaughn, T., Forrest, K., Kokomo-Awich, J., Smith, M., Law, M., Oredipe, O. and Dunston, G.M. Activated eosinophil conditioned supernatants modulate adhesion molecule expression on prostate tumor cells. Presented at the Meeting of Research Centers for Minority Institutions in Honolulu, HI, December 2002.

Furbert-Harris PM, Ellison GL, Laniyan I, Gause DP, Vaughn T, Forrest K, Dunston GM, and Rotimi CN. Association Between Tumor Necrosis Factor-alpha (TNFα) and Correlates of Insulin Resistance in a Cohort of Type 2 Diabetic Patients from West Africa. Presented at the Meeting of Research Centers for Minority Institutions in Honolulu, HI, December 2002.

Discussion/Conclusion

We have hypothesized that activated eosinophils will bind to, infiltrate tumor spheroids, release their granular contents and other products such as cytokines; that these products could either modulate adhesion molecule expression on the tumor cells, thereby preventing binding to and invasion of endothelium, or kill the spheroid from within. These studies thus far have demonstrated that eosinophils can bind to and infiltrate tumor spheroids with and without IL-5 activation. IL-5 treated eosinophils, however, were activated as evidenced by large vacuoles and more mature eosinophils, and also larger concentrations of release eosinophil peroxidase throughout the spheroid and along the spreading growth. From these studies, it is evident that the culture period of the tri-cell complex is insufficient to microscopically detect cellular toxicity at the core. Future studies will extend the culture period from 24hrs to 48, 72 and perhaps as long as 1 week. Failure of both metastatic tumor cell line MDA-MB-231 and HC-202 to form spheroids has slowed the studies somewhat. We did, however examine invasion of MDA-MB-231 as single cell combinations and observed that eosinophil presence inhibited their invasion. With regards to fluorescent staining of the tri-complex of cells and the apparent loss of integrity of the antibodies used, a second explanation of the results is worthy of mentioning. Mortensen et al (18) have have reported that human cancer cells are able to fuse endothelial cells to form hybrid cells and that these cells can display proteins and chromosomal markers characteristic of both parent cells. According to these investigators, fusions of human breast cells and endothelial cells in vitro in co-cultures of human breast and endothelial cells and in vivo in nude mice experiments. Hence, according to these investigators, there is a new type of cancer-endothelial cell interaction which may be of fundamental significance to the process of metastasis. It is therefore quite possible that we have stumbled onto the same phenomenon. (Because of the need to revise this report and had the opportunity to be a reviewer on this manuscript 1 year after our work was done, I thought it quite significant and to add to this report).

The MTS model has previously been used to examine interactions of tumor:endothelium. The study of eosinophils in this tri-cell MTS:endothelium complex is the first of its kind attempting to examine the role of eosinophils in cancer. Immunohisto-chemical analyses revealed granular proteins in the tumor spheroid milieu. Moreover, cytokines such as IL-4 and other cytokines such as TNFa have been reported to be preformed in the eosinophil granular cores. The studies have shown that IL-4 in combination with IL-1 was able to decrease surface expression of ICAM-1 and VCAM-1 molecules on the metastatic cell line MDA-MB-231. These adhesion molecules play a role in tumor metastasis. It is clear from these and other previous studies in our laboratory that eosinophils can actively inhibit tumor growth and possible metastasis, and that the mechanism of this inhibition may involve cytokines which are often preformed in the eosinophil and also other toxic eosinophil mediators. Additional studies need to be executed to better define this mechanism (and in particular the role of isolated eosinophil proteins), in cancer and cancer metastasis.

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Appendices

Figure 1. Cytospin Preparations of Eosinophil Cell Line Stained with (A) Hematoxylin and Eosin (B) Giemsa Maygrunwald (C) Luxol Fast Blue

Figure 1. Legend

Figure 2. Eosinophil 24hr Supernates

Figure 2. Legend

Figure 3. Eosinophil Cell Lines Produce Interleukin-5 I.

Figure 3. Legend

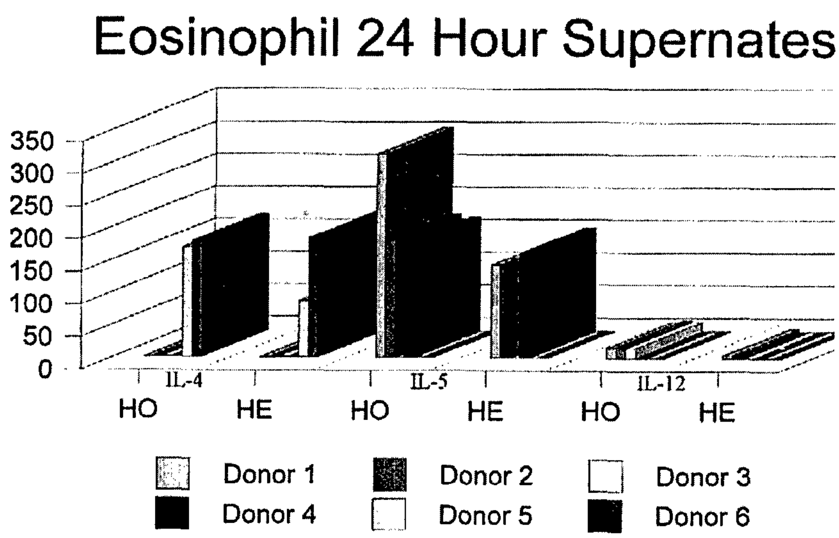
Figure 4. Presence of Interleukin-5 in Cultured Supernatants of Eosinophil Cell Lines II.

Figure 1. Cytospin Preparations of Eosinophil Cell Line stained with (A) Hematoxylin and Eosin (B) Giemsa May-Grunwald (C) Luxol-Fast-Blue

Figure 1. *Cytospin Preparations of Eosinophil Cell Line.* Eosinophil cell-lines stained with hematoxylin and eosin (A) giemsa maygrunwald (B) and luxol fast blue (C) vacuoles and granules can be readily seen.

Figure 2. Eosinophil 24 Hour Supernatants. Hypo- and hyperdense peripheral blood eosinophils from several donors with levels of allergic hypersensitivity 24hr at 37C in a humidified air conditions. Supernatants were harvested and tested by ELISA analysis for the presence of IL-4, IL-5 and IL-12.

Figure 2



HO - Hypodense Eosinophils
HE - Hyperdense Eosinophils

Figure 3. Eosinophil Cell Lines Produce IL-5 I. Hypo-dense and hyperdense esosinophil cell lines were cultured for 24hr and 72hr at 1X106 cell/ml. The supernatants were collected and examined for the presence of IL-5.

Figure 3

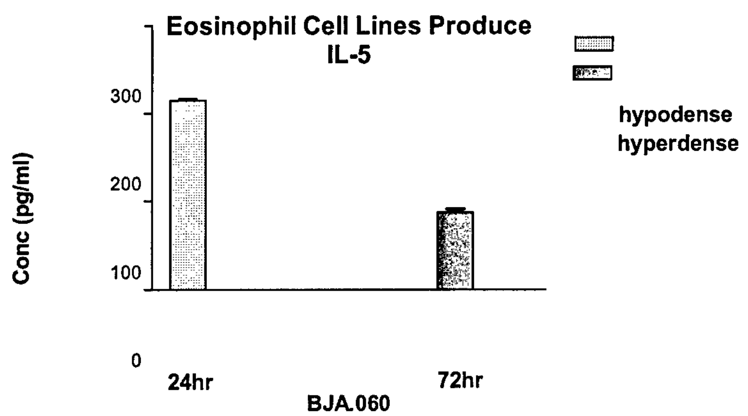


Figure 4. Presence of Il-5 in Cultured Supernatants of Eosinophil Cell Line II. Hypo- and hyperdense eosinophil cell lines established from peripheral blood eosinophils from an individual with severe allergic asthma and breast cancer, were cultured as described previously. IL-5 concentrations were determined by ELISA analysis.

Figure 4

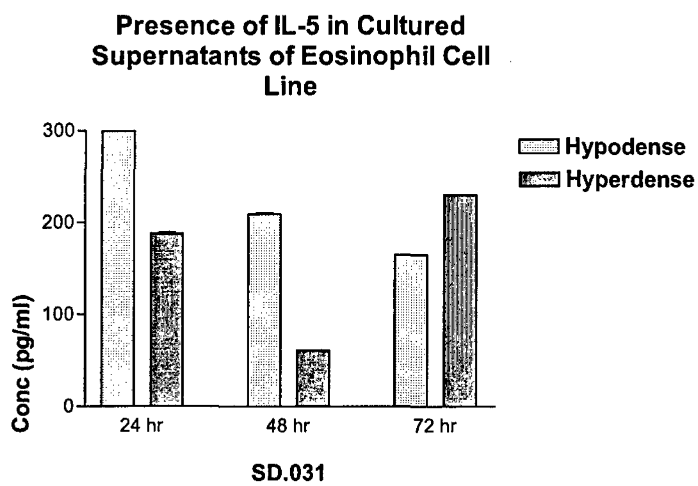
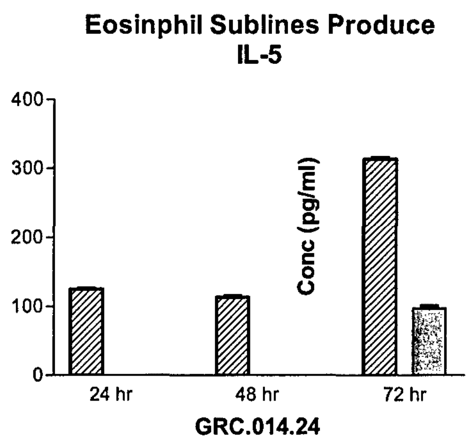


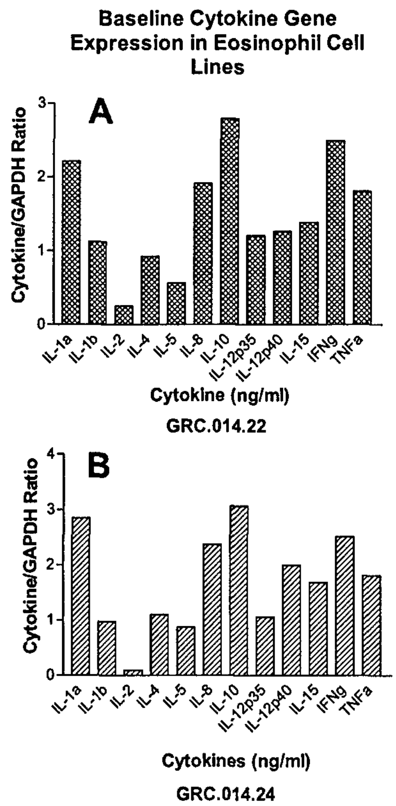
Figure 5



Eosinphil Sublines Produce
IL-5
400
300
200
100
0
Conc (pg/ml)
24 hr
48 hr
72 hr
GRC.014.24

Figure 5. ***Eosinophil Sublines Produce IL-5 III.*** **Hyperdense eosinphil cell line GRC.04.24 sorted using anti-eotaxin receptor antibody. The CCR3 positive cells were collected and put into culture, then frozen and reestablished in culture. This subline, GRC.014.24S1 was then sorted using VLA-4 (CD49D) as the marker. They were subcultured as described for the previous subline. All three lines (parent and 2 sublines) were cultured as described in the above figures for 24hr, 48hr and 72hr. The conditioned supernatants were examined for IL-5 by ELISA.**

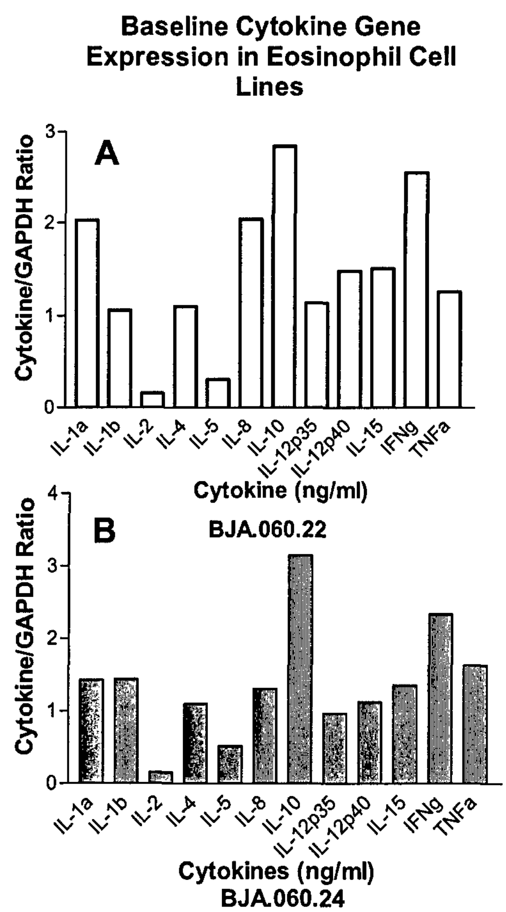
Figure 6



Baseline Cytokine Gene Expression in Eosinophil Cell Lines
A
Cytokine/GAPDH Ratio
0
1
2
3
IL-1a
IL-1b
IL-2
IL-4
IL-5
IL-8
IL-10
IL-12p35
IL-12p40
IL-15
IFNg
TNFa
Cytokine (ng/ml)
GRC.014.22
B
Cytokine/GAPDH Ratio
0
1
2
3
4
IL-1a
IL-1b
IL-2
IL-4
IL-5
IL-8
IL-10
IL-12p35
IL-12p40
IL-15
IFNg
TNFa
Cytokines (ng/ml)
GRC.014.24

Figure 6. ***Baseline cytokine gene expression in eosinophil cell lines I.*** **Total RNA from cell lines GRC.014.22 (A) and GRC.014.24 (B) was extracted using RNA purescript kits (Gentra Systems). The 2-step RT-PCR was performed, using 1 ug of RNA to first reverse tranxcribe to cDNA, for analysis of basal levels of cytokines. Messenger RNA of cytokines was estimated using the delta ct ratio of Cytokine/GAPDH (housekeeping gene). Eosinophil cell lines produce an array of cytokines at various concentrations.as seen with the plate of 12 in this assay. Concentrations of cytokine mRNA in the hypodense cell line (A) were slightly higher than in the hyperdense cell line (B).**

Figure 7



Baseline Cytokine Gene Expression in Eosinophil Cell Lines
A
Cytokine/GAPDH Ratio
0
1
2
3
IL-1a
IL-1b
IL-2
IL-4
IL-5
IL-8
IL-10
IL-12p35
IL-12p40
IL-15
IFNg
TNFa
Cytokine (ng/ml)
BJA.060.22
B
Cytokine/GAPDH Ratio
0
1
2
3
4
IL-1a
IL-1b
IL-2
IL-4
IL-5
IL-8
IL-10
IL-12p35
IL-12p40
IL-15
IFNg
TNFa
Cytokines (ng/ml)
BJA.060.24

Figure 7. ***Baseline cytokine gene expression in eosinophil cell lines II.*** **Total RNA from cell lines BJA.060.22 (A) and BJA.060.24 (B) was extracted as described in the previous figure (figure 6) Similar results sere seen with theses cell lines as with the previous 2 cell lines. Both sets of cell lines were developed from peripheral blood of moderately to severly allergic individuals. Again the hypodense cell line (A) generally produece higher levels of cytokine message than the hyperdense cell line (B).**

Figure 8. MCF-7 Multicellular Tumor Spheroids. MCF-7 multicellular tumor spheroids after three (A, C) and 7 (B, D) days in culture at 37C on 0.5% noble agar containing RPMI complete medium.

Figure 8.

MCF-7 Multicellular Tumor Spheroids

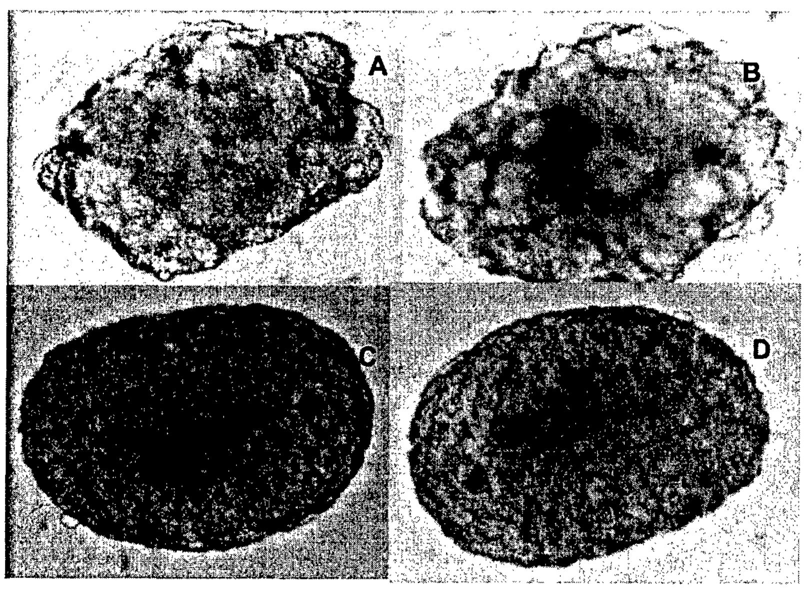


Figure 9. Effect of Interleukin-5 on MCF-7 Spheroid Growth. 7-day MCF-7 multicellular tumor spheroids spheroids were cultured in 10% RPMI complete medium (A) or with IL-5 at 0.5ng/ml (B), 1ng/ml ©), 2.5ng/ml (D), 5ng/ml (E) and 10ng/ml (F). The changes in spheroid growth were captured microscopically.

Figure 9

Effect of Interleukin-5 on MCF-7 Spheroid Growth

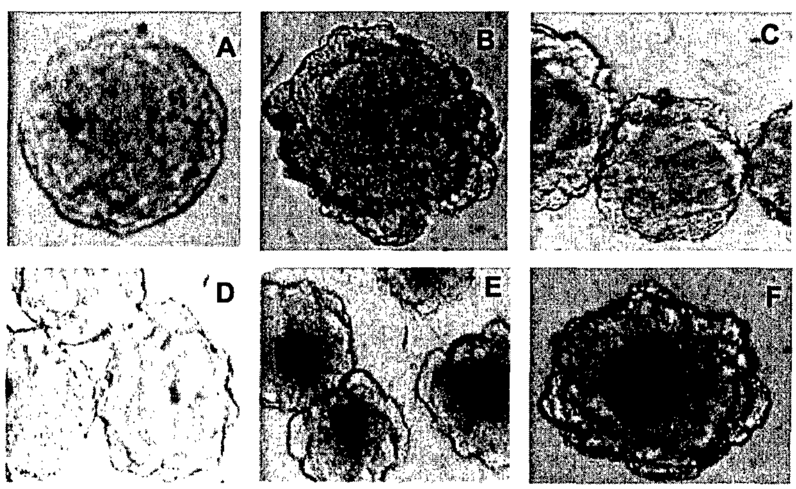


Figure 10. Effect of Interleukin-4 on MCF-7 Multicellular Tumor Spheroid Growth. 7-day MCF-7 multicellular turmor spheroids were cultured with 10% RPMI complete medium (A) and with IL-4 at 0.5ng/ml (B), 1ng/ml (C), 2.5ng/ml (D), 5ng/ml (E) and 10ng/ml (F). Growth of the spheroids were observed microscopically and photo documentation made.

Figure 10

Effect of Interleukin-4 on MCF-7 Multicellular Tumor Spheroid Growth

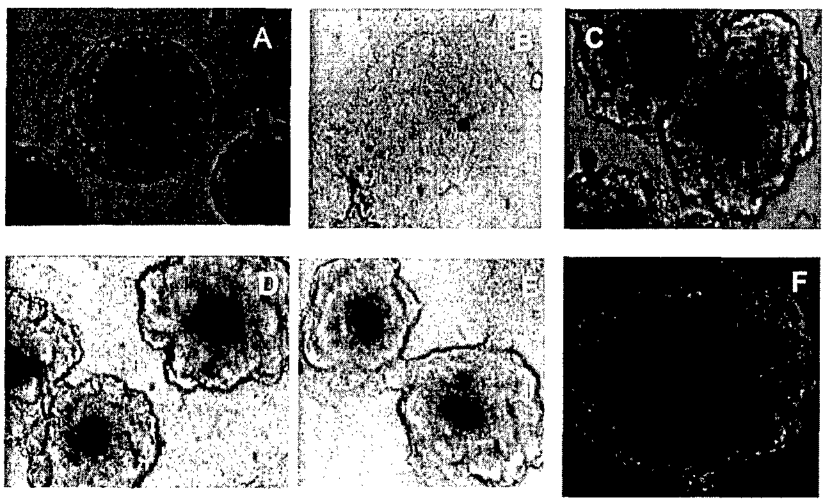


Figure 11. MCF-7 Multicellular Tumor Spheroid Binds to HUV-EC-C Monolayer. MCF-7 multicellular tumor spheroids cultured on human umbilical vein endothelial cell (HUV-EC-C) monolayer after 1hr (A) 24hr (B) and 48hr (C) culture. Some spheroids develop necrotic cores overtime (B). As the spheroid binds tightly to the HUV-EC-Cs, cells begin to grow out from the spheroid forming a monolayer over the HUV-EC-Cs (B, C) often destroying them overtime

Figure 11.

MCF-7 Multicellular Tumor Spheroid Bound to HUVEC Monolayer

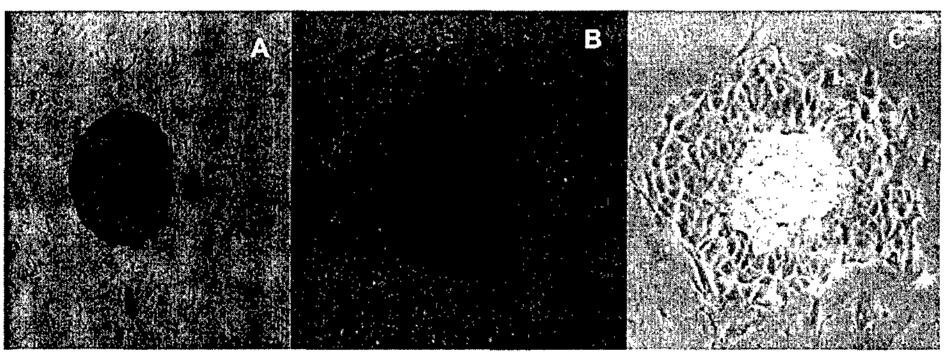


Figure 12.

Eosinophil:MCF-7 MTS Cocultures on HUVEC Monolayer

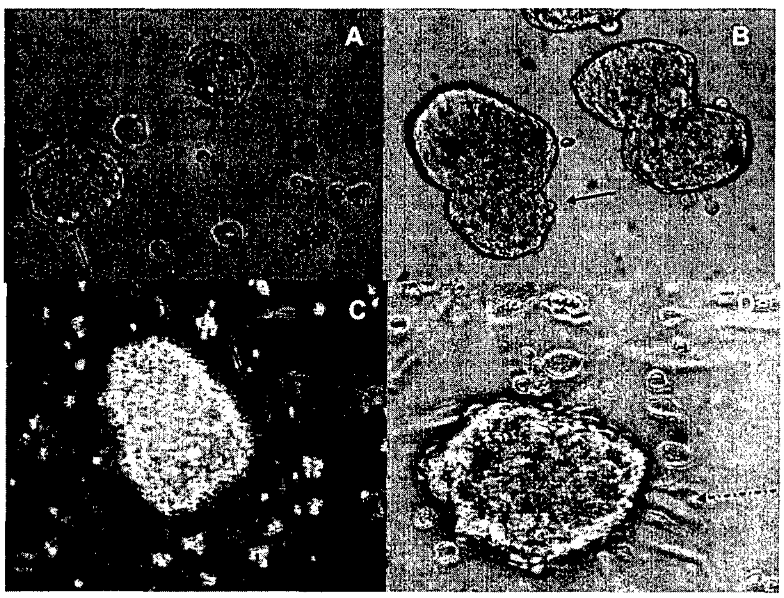


Figure 12. ***Eosinophil:MCF-7 MTS Co-cultures on HUV-EC-C Monolayer.*** **Eosinophils were first cultured with MCF-7 multicellular tumor spheroids for 24hrs then cultured on a lawn of HUV-EC-C cells. The solid arrows point to esoinophils bound tightly to the spheroids (A, B) as well as unbound eosinophil clusters (C). In figure 12C, the eosinophils completely surround the spheroid. Figure 12D shows high power resolution of an MCF-7 MTS with bound eosinophils attached to HUV-EC-C cells. The hatched arrow points to an endothelial cell.**

Figure 13. Histological Examination of Eosinophil:MCF-7 MTS on HUV-EC-C Monolayer (1hr co-culture). Eosinophils were cultured with MCF-7 MTS for 24hrs, then placed on a lawn of HUV-EC-Cs for 1 hr in a Falcon 8-chamber tissue culture slide. The slide was then washed with PBS, fixed with methanol, then stained with giemsa maygrunwald stain for the presence of eosinophils. Figure 13A shows an spheroid attached to HUV-EC-Cs. 24hr co-culture of eosinophils with MCF-7 spheroids resulted in the eosinophils binding

Figure 13 Histological Examination of Eosinophil:MCF-7 MTS on HUVEC Monolayer (1hr Coculture)

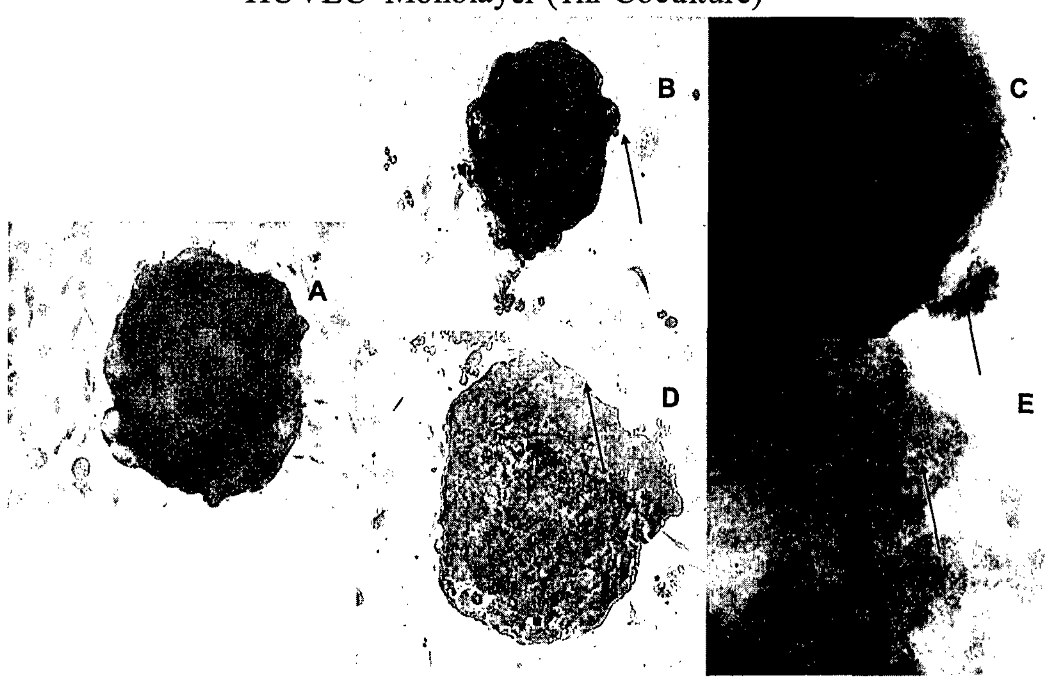


Figure 14. Histological Examination of 24hr IL-5 Treated Eosinophil:MCF-7 MTS on HUV-EC-C Monolayer (1hr co-culture). Eosinophil:MTS cultures were treated with interleukin-5 (IL-5) at 5ng/ml (24hr), then cultured on HUV-EC-Cs for 1hr. As was the case with the non-IL-5 treated Eosinophil:MTS co-cultures, eosinophils (larger numbers) can be seen attached to both the spheroids and the HUV-EC-C.

Figure 14. Histological Examination of 24hr IL-5 treated Eosinophil:MCF-7 MTS on HUVEC Monolayer (1hr Coculture)

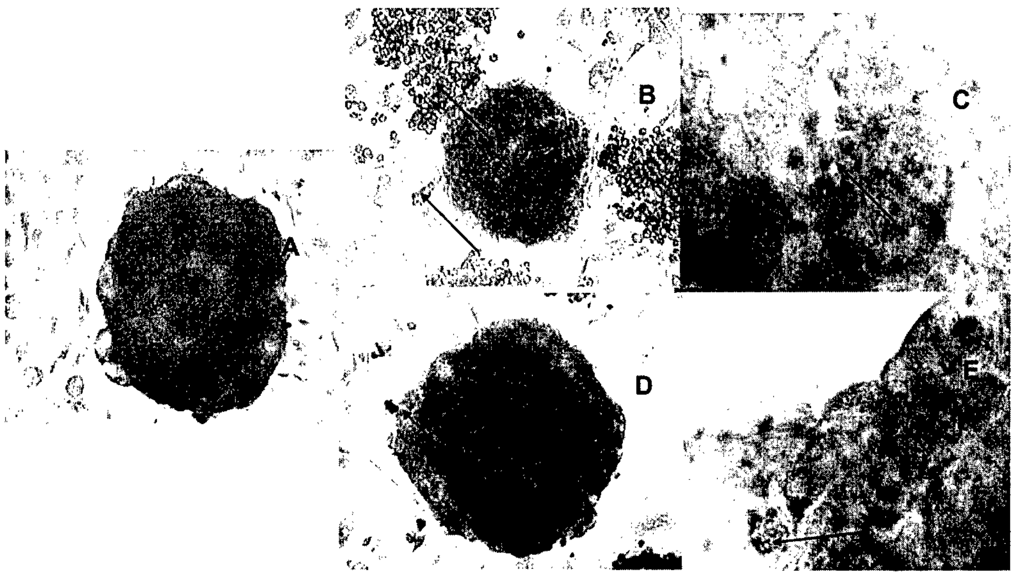


Figure 15. Histological examination of MCF-7 MTS and 24hr IL-5- treated eosinophils on HUVEC monolayer (1hr coculture). Eosinophils were incubated with IL-5 (24hr), then cultured with MTS and HUV-EC-C for 1 hr, the eosinophils were more activated and bound more readily to the HUV-EC-Cs, preventing the tumor spheroid from binding.

Figure 15 . Histological Examination of MCF-7 MTS and 24hr IL-5 treated Eosinophils on HUVEC Monolayer (1hr Coculture)

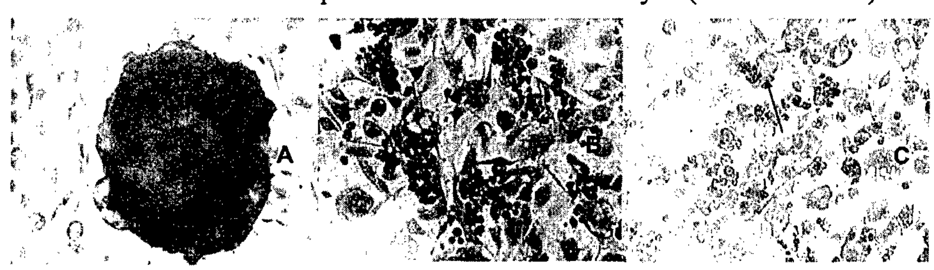


Figure 16. Histological Examination of Eosinophil:MCF-7 MTS on HUV-EC-C Monolayer (24hr co-culture). MCF-7 spheroids post 24hr culture with HUV-EC-Cs begin to spread out along the HUV-EC-C monolayer (fig. 16A). The presence of eosinophils on and within the spheroid does not affect the spreading of the tumor cells, however the eosinophils remain bound to the tumor cells as they spread out (fig. 16B, 16C, 16D). Eosinophils can be seen at the arrow heads and more clearly at 40X (fig. 16E) and 100X (fig. 16F) magnification, respectively. The hatched arrow is pointing to a tumor cell that has moved out from the spheroid with eosinophils attached (16E). 24hr cultures reveal more movement of tumor cells out from the spheroid and attachment of eosinophils (16B, 16C). Figure 16D and 16E are magnifications of 16B and 16C showing tremendous eosinophilic presence (arrows).

Figure 16. Histological Examination of Eosinophil:MCF-7 MTS on HUVEC Monolayer (24hr Coculture)

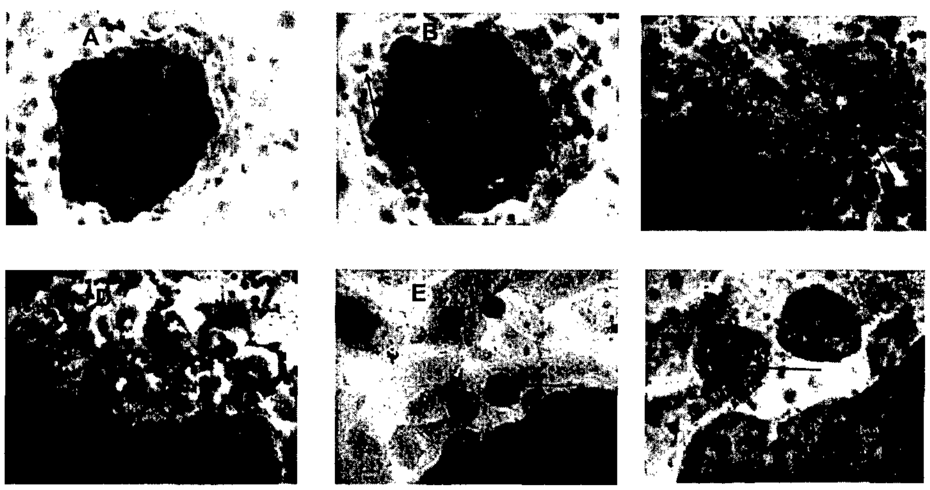


Figure 17. Histological Examination of 24hr IL-5 Treated Eosinophil:MCF-7 MTS on HUV-EC-C Monolayer (24hr co-culture). As was the case with the 1hr cultures, eosinophil-**infiltrated MCF-spheroids were pretreated with IL-5 (5ng/ml) then seeded onto HUV-EC-C monolayers which were further cultured for an additional 24hrs. Prior to co-culture, eosinophils were pretreated with IL-5 (5ng/ml) for 24hr.**

Figure 17. Histological Examination of 24hr IL-5 treated Eosinophil:MCF-7 MTS on HUVEC Monolayer (24hr Coculture)

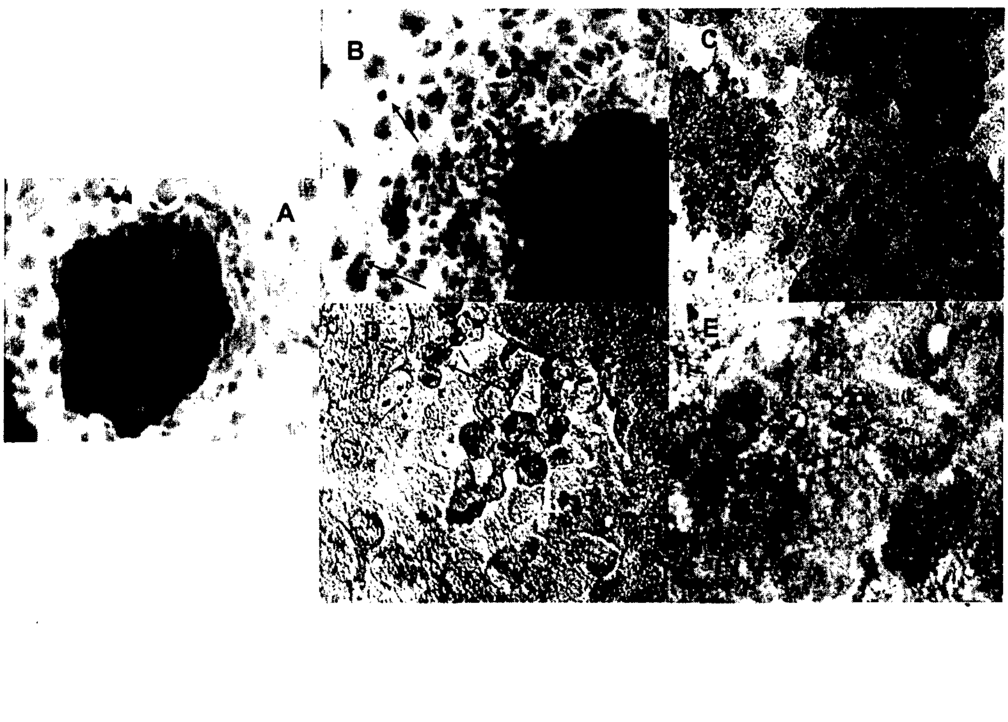


Figure 18. Histological Examination of MCF-7 MTS and 24hr IL–5 Treated Eosinophils on HUV-EC-C Monolayer (24hr co-culture). These data again show massive involvement of eosinophils with MCF-7 tumor spheroids and also with endothelial cells. IL-5 pretreatment of eosinophils induced greater activation of the eosinophils than the other two treatments (IL-5 treated eosinophils spheroids; IL-5 present in the culture medium with eosinophils and spheroids.

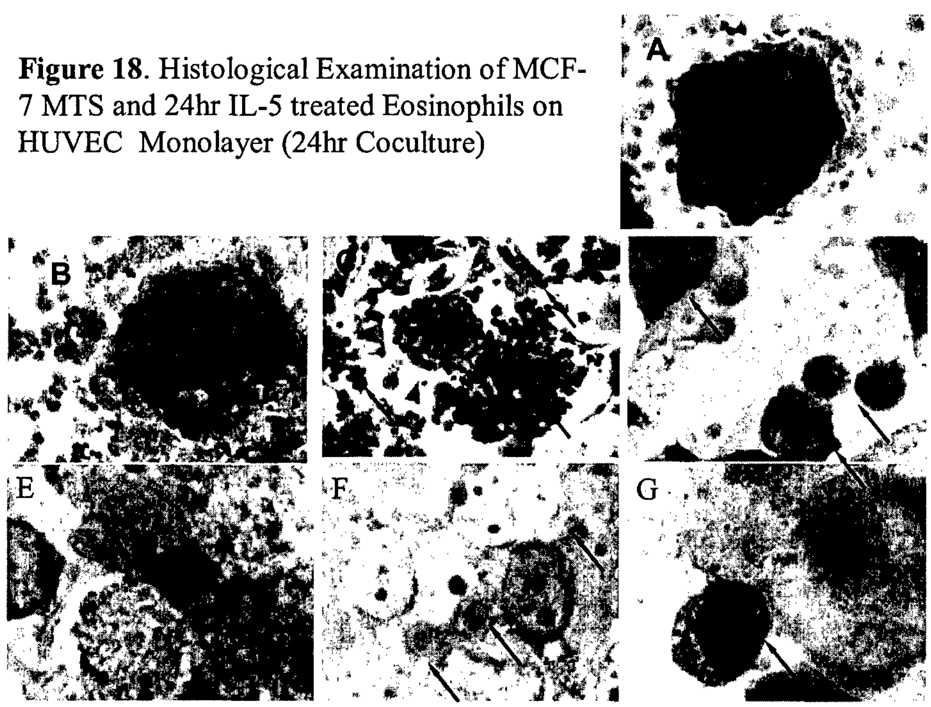


Figure 18. Histological Examination of MCF-7 MTS and 24hr IL-5 treated Eosinophils on HUVEC Monolayer (24hr Coculture)

Figure 19. Photomicrograph of Stained Section of Eosinophil:MCF-7 MTS (A) and Electron **Micrograph of Fine Section of Eosinophil:MCF-7 MTS (B). Eosinophil-infiltrated** spheroid was sectioned and stained with hematoxylin and eosin (fig 19A). The core of the spheroid contained many esoinophils and can be seen throughout the sectioned spheroid. Moreover transmission electron micrograph of a thin section of the spheroid was also prepared and again granulated esoinophils can readily be seen throughout the section (arrows, figure 19B).

Figure 19. A) Photomicrograph of stained section of Eosinophil:MCF-7 MTS. B) Electron micrograph of fine section of Eosinophil:MCF-7 MTS

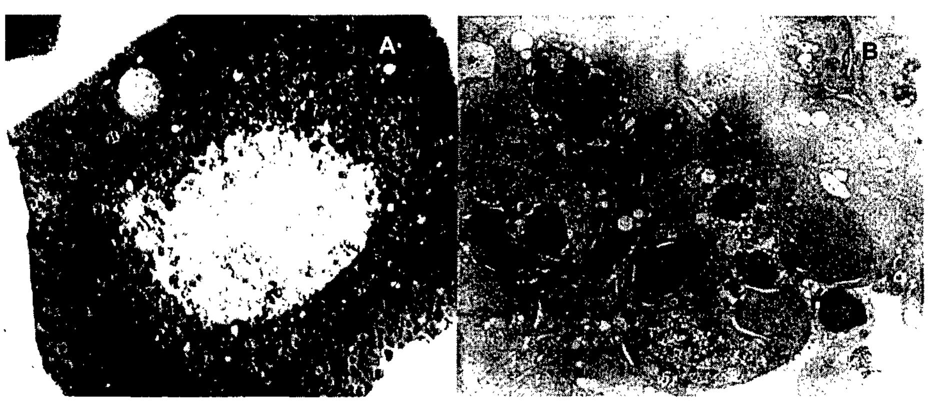


Figure 20. Fluorescent staining of Eosinophils, HUV-EC-Cs and MCF-7. Cells were stained individually with fluorescent dyes FITC, Texas Red and DAPI, respectively. The marker antigens used were Major Basic Protein (Eosinophils) von Willibrand Factor (HUV-EC-C) and Epithelial Cell Antigen (MCF-7).

Figure 20

Fluorescent Staining of Eosinophils, HUVEC, and MCF-7 Cells

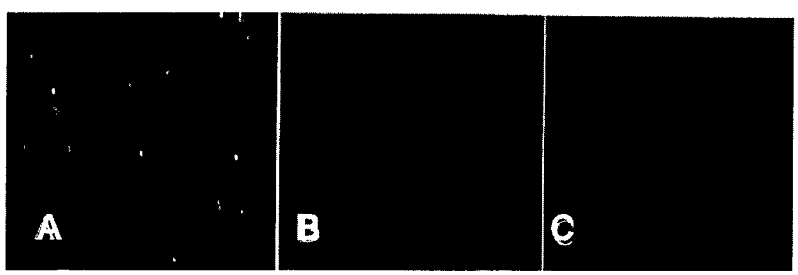


Figure 21. Dual staining of combinations of eosinophils, HUVEC, and MCF-7 cells. In 21 A the arrow points to an eosinophil binding to HUV-EC-C cells and in 21 B MCF-7 binding to HUV-EC-C.

Figure 21

Dual Staining of Combinations of Eosinoophils, HUVEC, and MCF-7 Cells

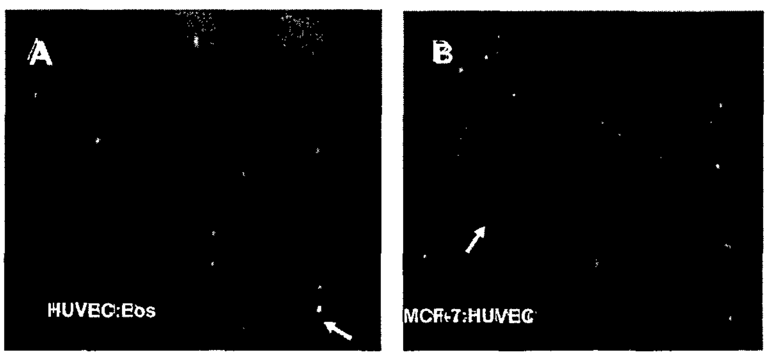


Figure 22. Triple staining of eosinophil:MCF-7:HUVEC tri-cell complex. MCF-7 tumor cells (lx103) were added to HUV-EC-C monolayers and incubated at 37C which hypodense eosinophils were added at 100:1 Eosinophils to tumor ratio. The plates were further incubated for 24hrs. The cultures were then stained with triple stain FITC, DAPI and Texas Red. The solid arrow points to an eosinophil and the hatched arrow points to a tumor cell, while the red cells are HUV-EC-Cs.

Figure 22

Triple Staining of Eosinophil:MCF-7:HUVEC
Tri-Cell Complex

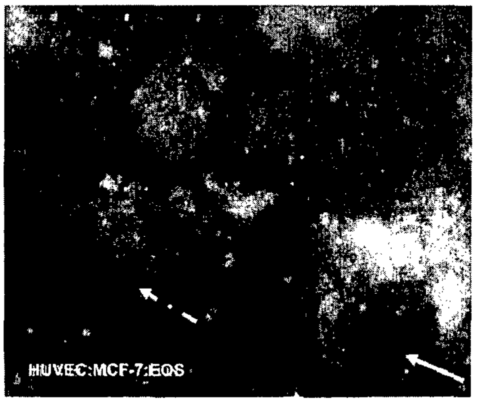


Figure 23. Photomicrographs of PKH26-Stained Eosinophils Co-Cultured with MCF-7 MTS. 7-day old MCF-7 MTS were cultured with hypodense eosinophils at 100:1 E.T ratio (200um MTS contains approximately lx105 cells) for 3 and 24hrs at 37C. The eosinophils had been pertained with the fluorescent vital stain PKH26. Figures A and C are phase contrast binding to or bound to the spheroid and in C the spheroid appears to be disintegrating. In figures B and D eosinophils can be seen bound (3hrs) and completely infiltrated the spheroid which had lost its integrity.

Figure 23

PKH26-Stained Eosinophils C0-Cultured with MCF-7 MTS

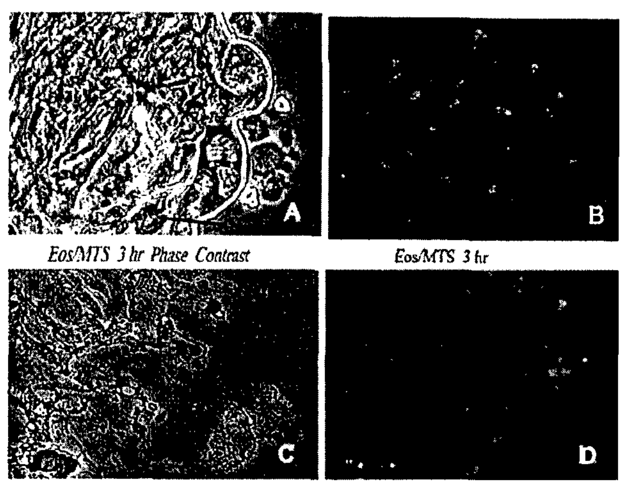
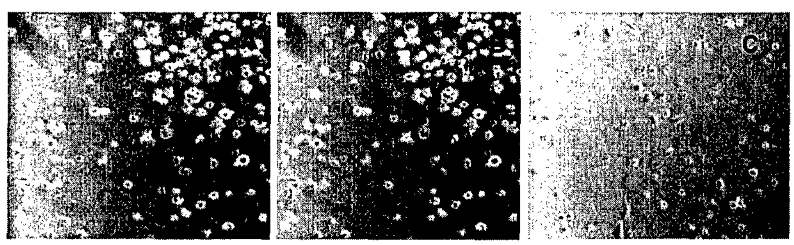


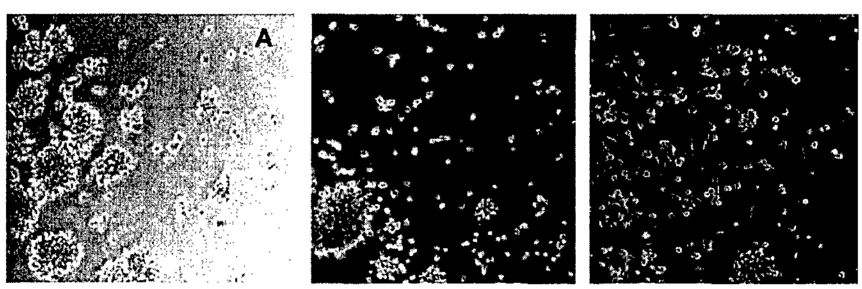
Figure 24. Invasion of HUVEC, MCF-7 and MDA cells. HUVEC cells were seeded onto the membrane of a transwell invasion chamber. The chamber was then incubated overnight at 37C. Tumor cells, MCF-7 (nonmetastatic) and MDA-MB-231 (metastatic) were added to the wells containing a lawn of endothelial cells. The chambers were incubated for an additional 24 hr. At harvest, the upper surface of the membrane was gently scraped using a cotton swab to remove all cells that did not invade the membrane:matrix complex. The membrane was carefully removed, using scalpel and forceps. Photomicrographs were taken of the underside of the membrane.

Figure 24.



Huvec Huvec + MCF-7 Huvec + MDA

Figure 25

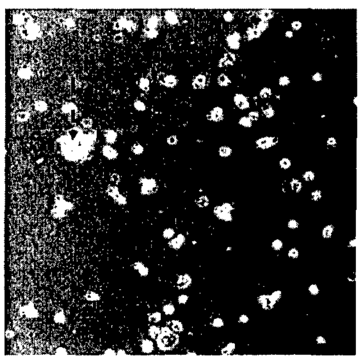


Huvec + Eos　　Huvec + MCF-7　　Huvec + MDA

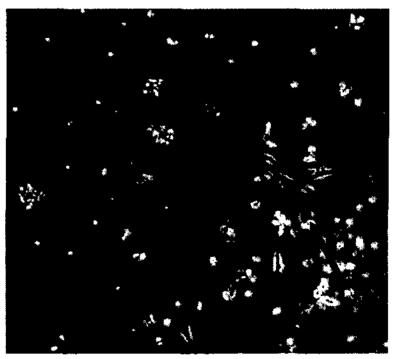
Figure 25. ***Invasion of eosinophil:huvec, eosinophil:tumor complexes.*** **HUVEC (A), MCF-7 (B) and MDA-MB-231 (C) cells were seeded onto the membrane of a transwell invasion system and incubated overnight. The next day 10% RPMI was placed in the lower chamber of the transwell invasion and eosinophils were added to the upper chamber on which a lawn of HUVEC endothelial cells or.tumor cells had grown. The transwell were incubated overnight. Upon harvesting the membrane was first scraped using a culture swab to remove any cells remaining and the under side was placed on a slide and captured microscopically.**

Figure 26. Eosinophils inhibit the invasion pattern of MDA-MB-231 tumor cells.HUVEC cells were seeded first onto the transwell membrane as previously described in figures 24 and 25. Secondly tumor cells, MCF-7 (A) and MDA-MB-231 (B) were added to the monolayer. The invasion plate was incubated for 2hrs followed by the addition of eosinophils. The dashed arrows point to the eosinophil clusters, the solid thin arrow points to the tumor cell and the thick arrow points to a HUVEC cell.

Figure 26.



Huvec+ MCF-7 + Eos

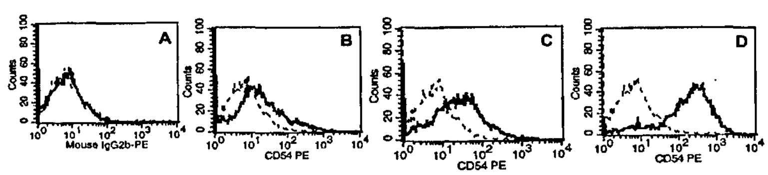


Huvec + MDA + Eos

Figure 27. The effect of IL-1 and IL-4 on ICAM-1 expression on MCF-7 tumor cells. ICAM-1 surface expression was determined pre- and post treatment with IL-1 (B) and IL-4 (C) by flow cytometry, using direct or indirect staining methods. Baseline expression can be seen in A and IL-1/IL-4 combination in D.

Figure 27.

The Effect of IL-1 and IL-4 on ICAM-1 Expression on MCF-7 Tumor Cells

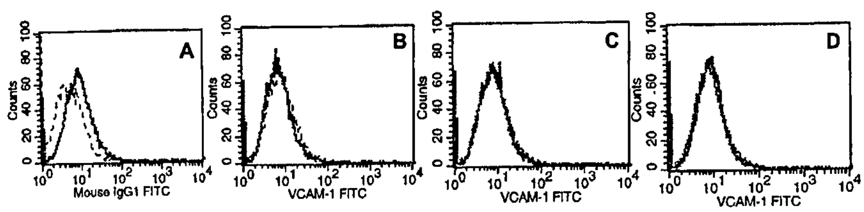


Log Fluorescence

Figure 28. The effect of IL-1 and IL-4 on VCAM-1 expression on MCF-7 tumor cells. VCAM-1 expression was determined for pre- and post IL-1 (B), IL-4 (C) and combination of IL-1 and IL-4 (D) by flow cytometry as described previously.

Figure 28

The Effect of IL-1 and IL-4 on VCAM-1 Expression on MCF-7 Tumor Cells

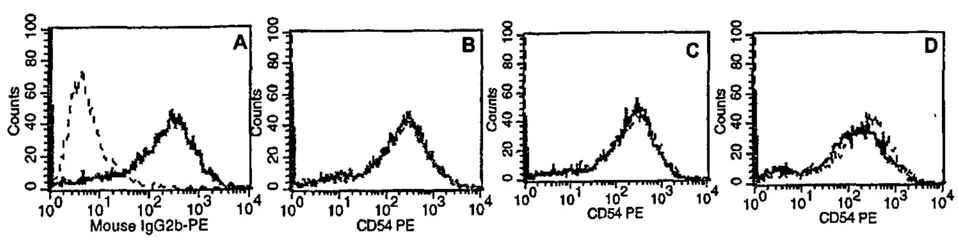


Log Fluorescence

Figure 29. The effect of IL-1 and IL-4 on ICAM-1 expression on MDA-MB-231 tumor cells. ICAM-1 surface expression on MDA-MB-231 tumor cells was determined by flow cytometry using direct or indirect staining methods .

Figure 29

The Effect of IL-1 and IL-4 on ICAM-1 Expression on MDA-MB-231 Tumor Cells

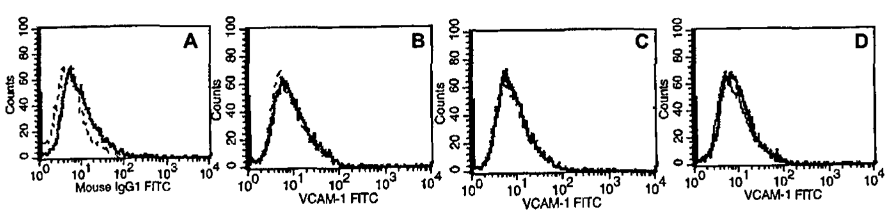


Log Fluorescence

Figure 30. The effect of IL-1 and IL-4 on VCAM-1 expression on MDA-MB-231 tumor cells. VCAM-1 expression was determined by Flow cytometry analysis. VCAM-1 is present on MDA-MB-231. IL-1 and IL-4 had no effect (B), however combination of IL-1 and IL-4 caused a slight decrease in surface expression (D).

Figure 30

The Effect of IL-1 and IL-4 on VCAM-1 Expression on MDA-MB-231 Tumor Cells

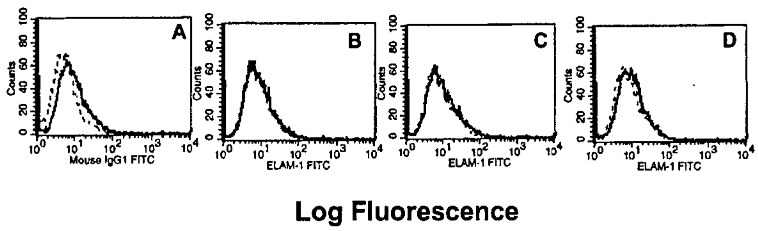


Log Fluorescence

Figure 31. The effect of Il-1 and IL-4 on ELAM-1 expression on MDA-MB-231 tumor cells. ELAM-1 expression was determined by flow cytometry analysis. MDA-MB-231 tumor cells were positive for ELAM-1. Neither IL-1, IL-4, alone or in combination affected its expression .

Figure 31

The Effect of IL-1 and IL-4 on ELAM-1 Expression on MDA-MB-231 Tumor Cells



ANTICANCER RESEARCH 23: 71-78 (2003)

Activated Eosinophils Infiltrate MCF-7 Breast Multicellular Tumor Spheroids

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Abstract. *Background: Previous studies in our laboratory have shown that activated eosinophils and eosinophilic cell lines inhibit the in vitro growth of MCF-7 breast tumor cells. We have also shown that IL-4 and IL-5 partially inhibit MCF-7 growth in vitro. In this study MCF-7 multicellular tumor spheroids (MTS) were developed to study the effect of eosinophils and IL-4 on tumor growth. Materials and Methods: Hypo- and hyperdense metrizamide density gradient fractions of eosinophils from peripheral blood of individuals with mild to moderate esoinophilia were co-cultured with 2-day-old MCF-7 MTS in medium containing bacto agar overlay at 37°C. Results: Light microscopic analyses revealed the attachment of eosinophil effector cells to the spheroid borders. Moreover, the culture media was greater than 90% devoid of effector cells. At six days post co-culture, very large spheroids were observed in both test and control dishes; however the necrotic cores in the co-cultures were more intense and larger than in the control. When MCF-7 tumor cells (1×10^6) were pretreated with IL-4 at 0.5 ng/ml, there was a dramatic decrease in the number of spheroids formed. Conclusion: These data strongly indicate that cytokines like IL-4 and perhaps other eosinophil mediators are capable of killing and inhibiting tumor growth; they suggest that tumor infiltrating eosinophils can degranulate and release toxic inhibitory factors into the tumor milieu which destroy the surrounding tumor. These observations, along with the use of the eosinophil: MTS tumor model provide a unique model system for in depth studies of the role of eosinophils and the cytokines they produce in breast cancer and may offer potential therapeutic implications.*

Eosinophils play a significant role in allergic and asthmatic inflammatory responses (1-5). Moreover, they are the primary effector cells in anti-helminthic infections (2, 3, 6, 7), and are also prominent in other disease conditions such as cutaneous hypersensitive disorders (8) and inflammatory bowel disease (9). Eosinophils have also been found in tumor cellular infiltrates and in peripheral blood, particularly post immunotherapeutic regimens (10-12). However, while their precise role in cancer remains equivocal, they do produce and release numerous mediators and cytotoxic molecules, such as major basic protein (MBP) and eosinophil peroxidase (EPO) which have direct toxic activity on tumor cells (13-15) and others (Leukotrienes, EPO) which indirectly enhance the production of toxic cytokines (16, 17). Eosinophils themselves produce an array of cytokines, some of which regulate their growth and differentiation in an autocrine fashion (*e.g.,* IL-3, IL-5, GM-CSF) (18, 19) and others (*e.g.,* IL-4, TNFa, IL-12) which may exert direct tumor growth inhibition as well as immunomodulatory activities (20-22).

The prognostic significance of eosinophils at tumor sites is still somewhat controversial. Some studies show positive correlation with good prognosis (10, 11, 23-25), while others indicate a harmful role in the pathophysiology of cancer or no association at all (26-29). There have been few studies on the biological activity of eosinophil anti-cancer activity *in vitro.* Kudo *et al.* showed that the eosinophil granular protein, MBP, was toxic to the erythroleukemic cell line, K562 (13), while Maeda's group demonstrated cytostasis with ECP on several human cell lines, including the breast lines MDA-MB-453 and T47D (30). In this study, we have utilized multicellular tumor spheroids (31, 32) to investigate the intimate *in vivo* interaction of eosinophils with tumor and the resultant impact on growth.

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Key Words: Eosinophils, multicellular tumor spheroids, breast cancer, cytokines.

Materials and Methods

Eosinophils. Peripheral blood leukocytes (PBLs) from eosinophilic allergic and asthmatic individuals were fractionated on a metrizamide density gradient (33). Hypodense (metrizamide fraction 22) and hyperdense (metrizamide fraction 24) eosinophilic fractions were collected, washed and counted.

Eosinophil Inhibition of MCF-7 Growth

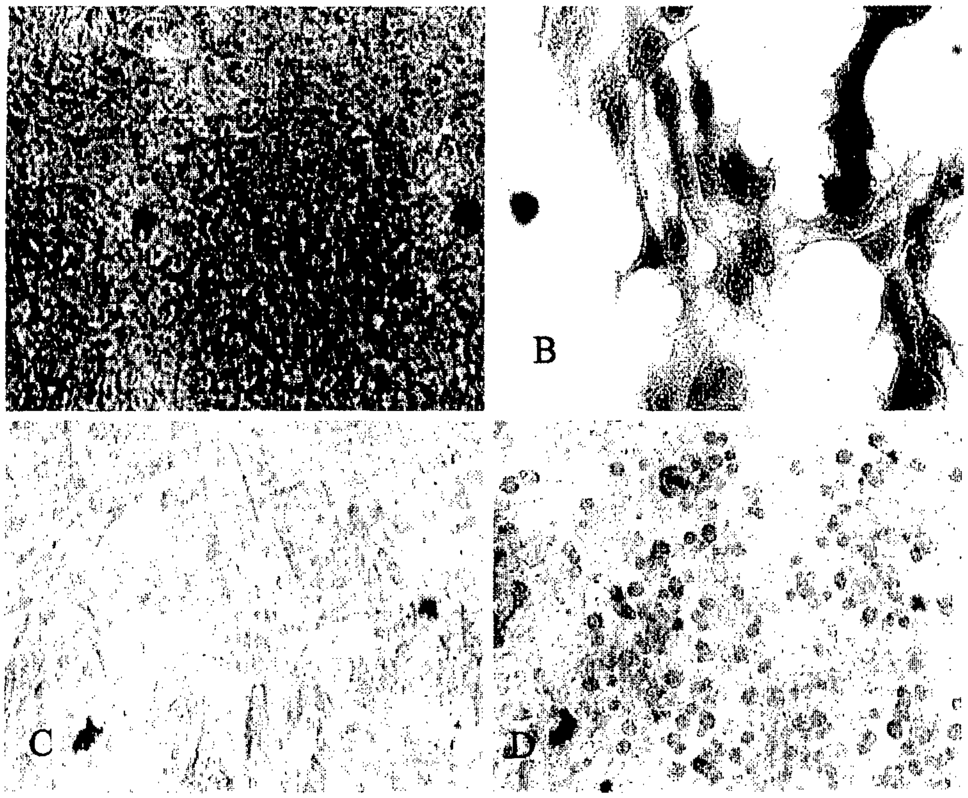


Figure 1. *MCF-7 tumor cells were seeded at 5 x 10^5/well and allowed to grow to confluency with media alone (A) or in the presence of activated eosinophils at E:T ratio of 2:1 (B). MCF-7 cells were also cocultured with eosinophilic cell lines formed from hypodense (C) and hyperdense (D) metrizamide fractions at the E:T ratio of 10:1.*

Monolayer coculture assay. MCF-7 tumor cells (obtained from ATCC, West Virginia and maintained in Eagle Minimum Essential Medium with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids and 1.0 mM sodium pyruvate and supplemented with 0.01 mg/ml bovine insulin, 90%; fetal bovine serum, 10%; temperature, 37°C) were seeded into the wells of either a 6-well plate (5 x 10^5 cells per well) or a 12-well plate (at 1.5 x 10^5 cells per well). The plates were incubated overnight (16-24 hours) at 37°C, 5% CO_2 incubator. Eosinophils were added at various effector to target (E:T) ratios and the plates were incubated for an additional 48-72 hours (when the control wells reached confluency). Cytokines were also added at various concentrations and the plates incubated for 72hours. The effector cells and supernatants were removed at the end of the incubation period, the monolayers were washed three times with PBS and the wells stained with hematoxylin and eosin. Photomicrographs of monolayer destruction were prepared and also monolayer growth inhibition was quantified by Integrated Density Value (IDV) measurement, using the Chemi-Imager 4000 (Alpha Innotech Corp., San Leanddro, CA, USA). Briefly, the well area was selected and saved as the spot overlay. This spot overlay was used on each well of a plate in order to standardize the area of all wells. As wells were selected, their IDV was automatically calculated. The IDV is the sum of all pixel values after background correction. Auto background correction uses the Alpha Ease program which determines the average of the 10 lowest pixel values in each individual well and assigns that value as background. However, if no background is selected the background value is reported as zero. Because the average (AVG) is equal to IDV divided by the overlay value (which is constant), we used AVG as a comparative figure. We then compared the IDV of the control with that of the test samples. The percent inhibition was calculated as follows:

Interleukin-4 and Interleukin-5 Inhibit MCF-7 Tumor Cell Growth

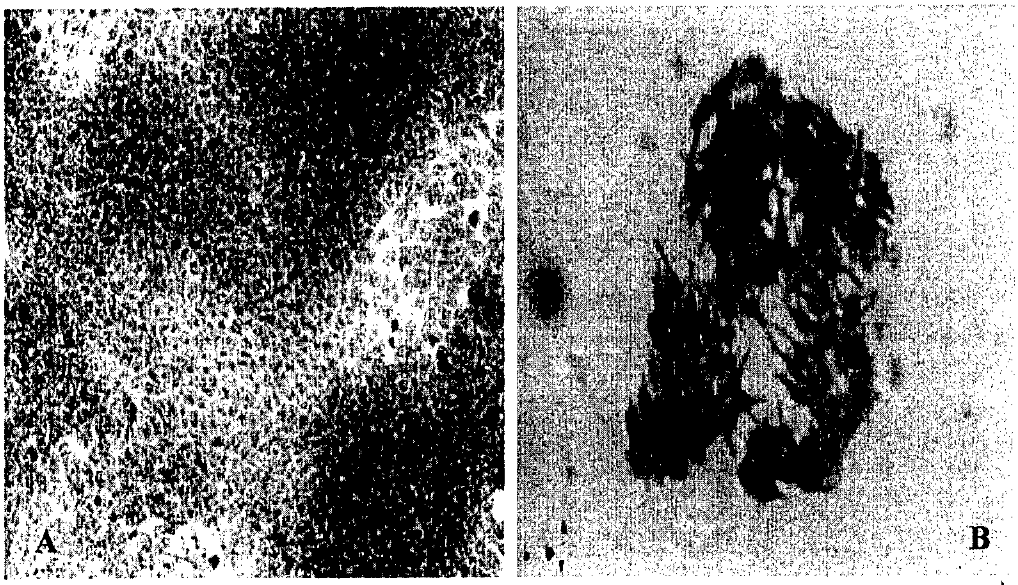


Figure 2. *Subconfluent MCF-7 cells in 6-well tissue culture plates were cultured for 4 to 5 days in media alone (A); media + IL-4 (B).*

$$\% \text{ Inhibition} = \frac{\text{average IDV (Control)} - \text{IDV (test)}}{\text{average IDV (Control)}} \times 100$$

MTS production. MCF-7 multicellular tumor spheroids were developed by slightly modifying the method of Yuhas *et al.* (31). Briefly, subconfluent monolayer cultures, maintained (at 37°C, 100% relative humidity, 95% air, 5% CO_2) in 10% RPMI complete medium were used to prepare MTS. After trypsinization and cell count, the cells were dispensed into T_{25} non-vented flasks (1 x 10^6 cells/flask), pulsed with CO_2, then capped and placed at 37°C in a rocking incubator and agitated at 30 periods per minute for 24-48hours. The spheroids were then transferred to 100-mm Petri dishes containing an overlayer of 0.5% noble agar in 10% RPMI medium. The dishes were then incubated at 37°C in a 5% CO_2 atmosphere for 7-14 days with regular feeding to study the growth characteristics of the MTS. Diameter measurements were made with an ocular micrometer on an inverted phase contrast microscope. At 48 hrs, spheroid diameters ranged in size from 250-800 μm.

Eosinophils: MTS coculture assay. Five-10-day Eosinophil : MCF-7 MTS coculture assays were performed in 6-well tissue culture plates containing 0.5% bacto agar overlays. Five spheroids were placed in each well and eosinophils were added at effector to tumor (E:T) ratios of 10:1, 100:1 and 1,000:1. At the end of the culture period the MTS were collected and fixed with 3% glutaraldehyde for TEM analyses and in Karnovsky's fixative (4% depolymerized paraformaldehyde, 2% glutaraldehyde in 0.12M PBS, pH 7.4) for SEM analyses.

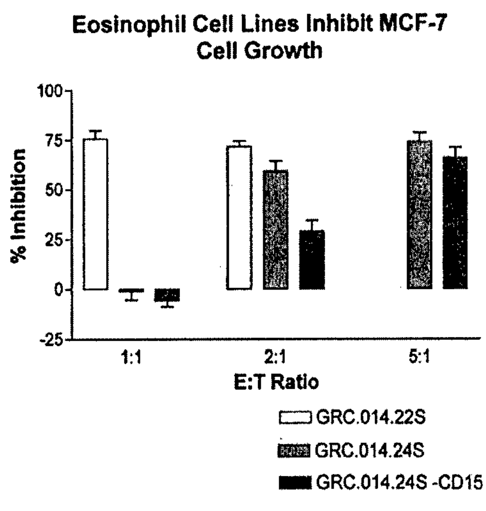
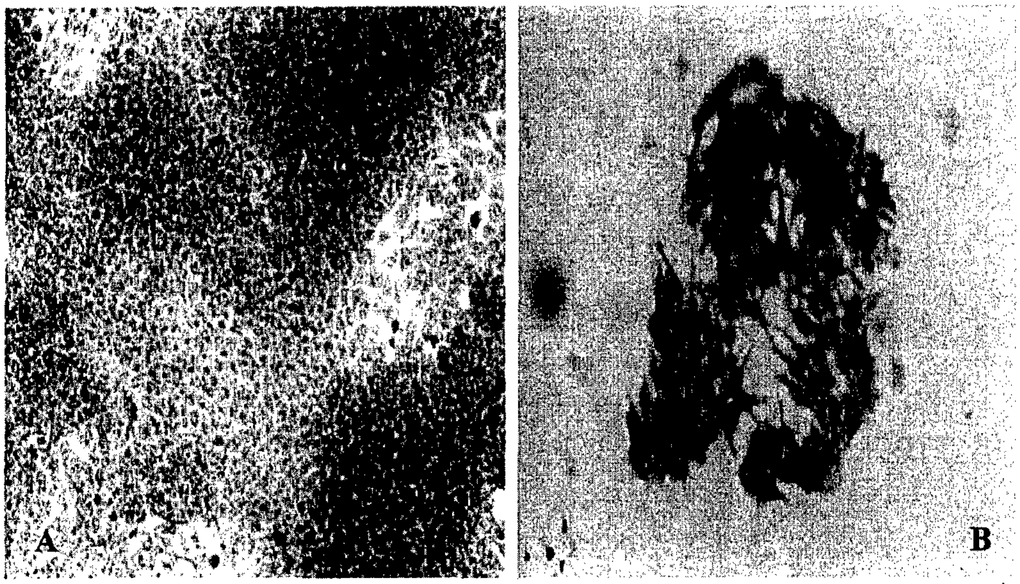


Figure 3. *Eosinophils cell lines were cultured with MCF-7 tumor cells for 48-72hours. Varying E:T ratios the monolayers were washed three times with PBS and stained with H & E. Density determinations were made and percent inhibition calculated as described in Materials and Methods.*

Interleukin-4 and Interleukin-5 Inhibit MCF-7 Tumor Cell Growth



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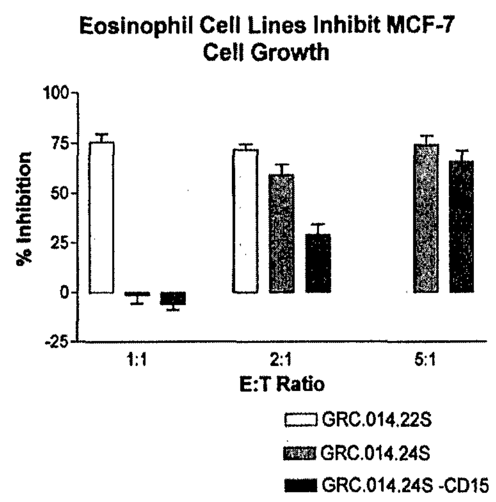
Eosinophils: MTS coculture assay. Five-10-day Eosinophil : MCF-7 MTS coculture assays were performed in 6-well tissue culture plates containing 0.5% bacto agar overlays. Five spheroids were placed in each well and eosinophils were added at effector to tumor (E:T) ratios of 10:1, 100:1 and 1,000:1. At the end of the culture period the MTS were collected and fixed with 3% glutaraldehyde for TEM analyses and in Karnovsky's fixative (4% depolymerized paraformaldehyde, 2% glutaraldehyde in 0.12M PBS, pH 7.4) for SEM analyses.



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Photomicrograh of Stained Section of MTS

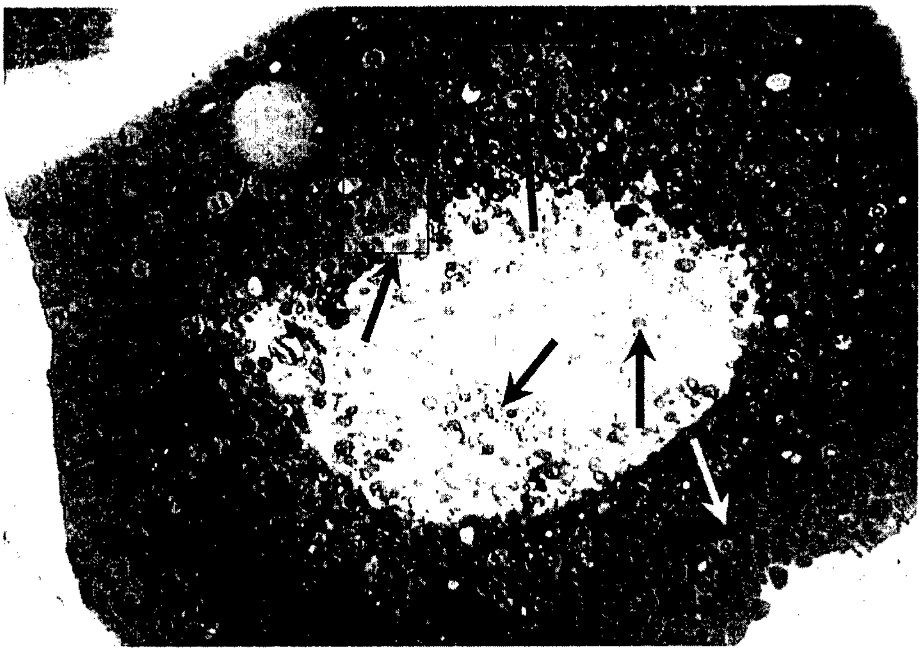

Figure 5. *Transverse section of MCF-7-MTS that has been infiltrated by eosinophils. The light area represents the core (seen in Figure 3 as the darkened necrotic core). Eosinophils are seen along the section and in the core. The boxed areas were magnified and can be seen in Figure 5.*

Photomicrograh of Stained Section of MTS

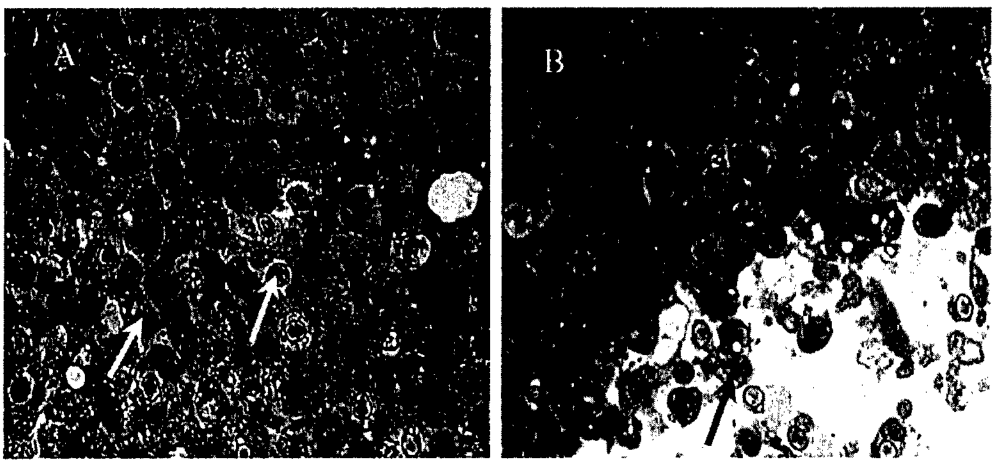

Figure 6. *A) is a magnification of the transverse section and B) is a magnification of the interface of the core and the transverse section. Granulated and highly vacuolated eosinophils can be readily seen.*

Electromicrograph of Eosinophil Infiltration of MCF-7 MTS

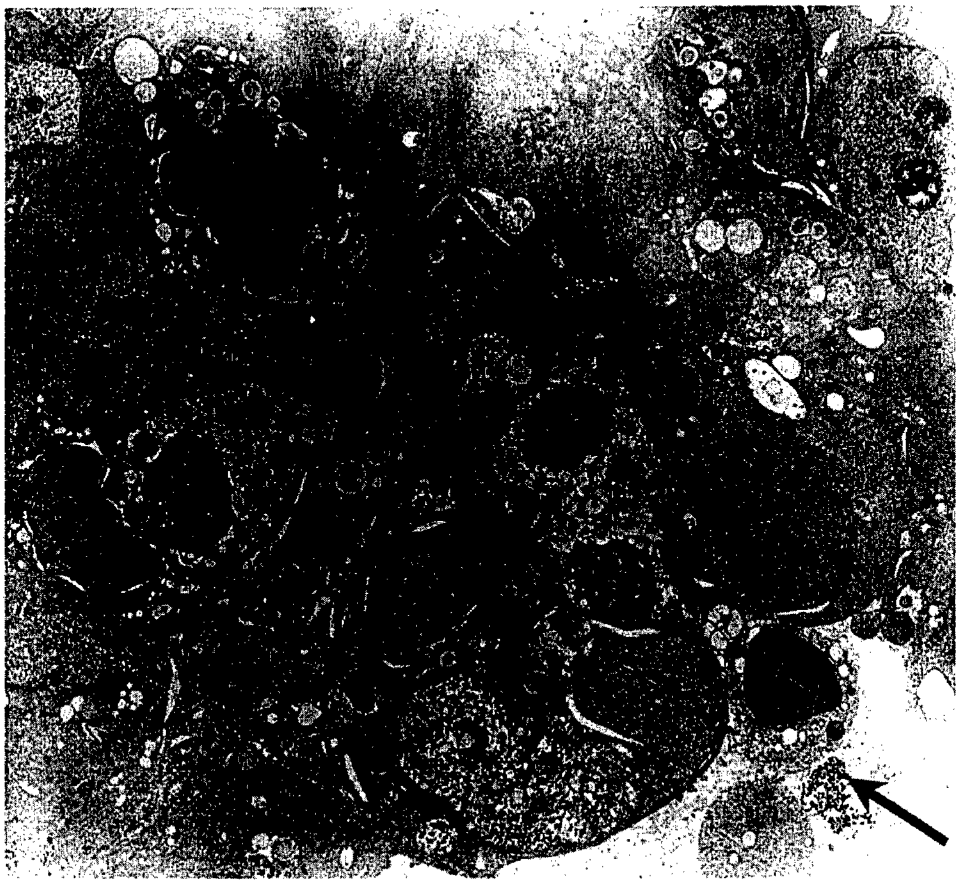

Figure 7. *Ultrastucture of hypodense eosinophil in MCF-7-MTS, 39K magnification. Granules and vacuoles can be seen.*

coculture of MTS with hypo-and hyperdense eosinophils (B). In C and D, bound eosinophils can be seen at the borders of the spheroids. The core of the MTS cocultured with hypodense eosinophils was much larger and more necrotic than with the hyperdense eisonophils.

Transverse section of MTS cultured with hyperdense eosinophils. Post coculture with eosinophils, glutaraldehyde fixed spheroids were stained with hematoxylin and eosin. Figure 5 is a photomicrograph of one MTS section. The light central area is the core and is filled with large numbers of eosinophils and eosinophils can be seen throughout the spheroid. We then selected two areas, one within the transverse region (large arrow) and one at the edge of the core and the transverse region (small arrow) to further magnify. This can be seen in Figure 6. Highly granulated eosinophils can be seen in the interspaces of tumor cells (6A arrows). Large vacuoles can be seen in some of the eosinophils (6B arrow). Transmission electron microscopic analysis (Figure 7) confirmed the observations seen with the light microscope, *i.e.*, the association of vacuolated eosinophils (thin arrow) with the tumor cells and highly dense granules both within the eosinophils cytoplasm (thick arrow) and released into the core (short arrow).

Discussion

Eosinophils are well studied as multipotential, multifunctional nonspecific immune cells which have tremendous immunoregulatory capacity. The controversy around the prognostic value of these cells in cancer involves the indiscriminate toxicity of their released granular proteins and the potential antagonism of immunomodulatory cytokines and that these together may collectively promote tumor growth. Although cytokines like IL-10 may down-regulate the immune response (34), others such a IL-12 are known to inhibit cancer cell mobility and invasion (35); and both are produced by activated eosinophils (36, 37). Eosinophilic mediators and cytokines also modulate adhesion molecule expression on endothelium and epithelial tissue, thereby allowing the trafficking and influx of immune and other inflammatory cells into sites of action (38), and may also induce adhesion molecule expression on tumor cells, thereby promoting their mobility and potential metastasis.

The results in this study have clearly shown that activated eosinophils obtained from peripheral blood of allergic and/or asthmatic individuals kill and prohibit the growth of MCF-7 breast tumor cells *in vitro,* but had minimal affect on the human fibroblast cell line MRC-5 (data not shown). Light and electron microscopic analyses of the eosinophil: MTS coculture have very dramatically demonstrated the attachment and infiltration of eosinophils into the core of the spheroid. As the eosinophils are transversing the spheroid there is potential for adhesion and rolling along the tumor cell surfaces, similar to eosinophil infiltration into any inflammatory site, including tumor tissue. Early studies on MTS demonstrated inherent necrosis at the core of the spheroid (31), however, interaction with eosinophils in this study resulted in a more rapid development of and more intense necrosis at the core. This eosinophil: MTS model (which has not been used before in this manner) allows one to better study and define the components released by eosinophils, the adhesion molecules involved in eosinophil binding to the tumor cells, (and molecules on the tumor that might be involved in growth and metastasis) as well as the expressed cytokine involvement. This model may potentially elucidate the role of eosinophils in cancer development and metastasis. These studies are currently being carried out and strategies are being designed to modulate eosinophils and their granular contents in anti-cancer activity.

Acknowledgements

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ACTIVATED EOSINOPHILS UPREGULATE THE METASTASIS SUPPRESSOR MOLECULE E-CADHERIN ON PROSTATE TUMOR CELLS

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Abstract - Cell adhesion molecules (CAMs) play an important role in cancer metastasis by facilitating attachment to vascular endothelia, invasion and spread into secondary tissue sites. We have shown that activated eosinophils (EosA) inhibited the growth of prostate cancer (Pca) cells *in vitro*. In the present study, we examined the ability of EosA 24 hr conditioned supernatants (EosAcs) to modulate the expression of ICAM-1, VCAM-1, ELAM-1, E-cadherin and N-cadherin expression on human Pca cell lines, Du-145 and PC-3 by flow cytometry. TNF-α, IL-10 and IL-12 were also evaluated. ICAM-1, expressed on PC-3 and DU 145 cells, was enhanced by TNF-α and IL-10. ELAM-1 was present on DU 145 cells but absent on PC-3. TNF-α and IL-10 enhanced ELAM-1 on DU 145, but EosA 24 hr supernatants failed to do so. All three cytokines, namely IL-10, IL-12 and TNF-α-induced ELAM-1 on PC-3 tumor cells. Although VCAM-1 was absent on DU 145 and PC-3 cells, it was expressed on DU-145 cells after exposure to EosA: tumor cell co-cultures, and was expressed on PC-3 following exposure to IL-10 and IL-12. N-cadherin and E-cadherin were both expressed on DU-145. While N-cadherin was expressed on PC-3 cells, E-cadherin was not. N-cadherin was enhanced on DU-145 and PC-3 cells following exposure to EosA co-culture and upregulated on PC-3 by IL-10 and EosA 24 hr supernatants, but decreased by IL-12. E-cadherin was up-regulated on DU 145 cells following co-culture with EosA and was induced on PC-3 by IL-10 and IL-12, but not by EosA co-culture and 24 hr supernatants. In conclusion, inflammatory and non-inflammatory cytokines modulate CAM expression on Pca cells; EosA and EosA 24 hr supernatants also exerted modulatory activity of CAM expression. Most significantly, the metastasis suppressor molecule, E-cadherin was enhanced on DU 145 cells by EosA and induced on PC-3 by IL-10 and IL-12 both of which are produced by EosA. This suggests potential use of these cytokines in immunotherapeutic strategies for prostate cancer and its metastasis.

Key words: Activated eosinophils, E-cadherin, interleukin-10, interleukin-12, ELAM-1, VCAM-1, ICAM-1, N-cadherin

Abbreviations: **EosA**: activated eosinophils; **EosA-CS**: activated eosinophil conditioned supernatant; **CAM**: cell adhesion molecules; **ICAM-1**: intercellular adhesion molecule-1; **ELAM-1**: extracellular adhesion molecule-1; **VCAM-1**: vascular cell adhesion molecule-1; **Pca**: prostate cancer; **VLA-4**: very late antigen-4; **VLA-6**: very late antigen-6; **E-cadherin**: epithelial cell adhesion molecule; **P-cadherin**: placental cell adhesion molecule; **N-cadherin**: neural cell adhesion molecule; **IL-10**: interleukin-10; **IL-12**: interleukin-12; **MMP-2**: matrix metalloproteinase-2; **SCID**: severe combined immunodeficiency; **TIMP-1**: tissue inhibitor of matrix metalloproteinase1; **TNF-α**: tumor necrosis factor alpha

INTRODUCTION

Prostate cancer is the most commonly diagnosed cancer in American men. It is second to lung cancer as the leading cause of male cancer deaths (2). The majority of prostate cancer deaths result from metastasis to surrounding tissues, blood and lymph circulation, and bone (15,16). While local cancer, which is confined to the prostate gland can be successfully treated with surgery, when the cancer breaks through the confines of the gland to invade and metastasize, treatment options are little to none (14,31,34,57). Metastasis is a process of many

complex steps during which cells detach from the primary tumor bind to the endothelium or extracellular matrix, and set up secondary foci at distant sites (1,21).

Like many other cells in the body, tumor cells express surface adhesion molecules which assist in cell:cell and cell:extracellular matrix interactions. These adhesion molecules, which are involved in cell motility, invasion and metastasis, include: a) intercellular adhesion molecules (e.g. ICAM-1, ICAM-2, C-CAM, V-CAM), b) substrate adhesion integrins (e.g. LFA-1, VLA-4, VLA-6), and c) calcium-dependent cadherins, of which there are many (17,29,48,50,59). E-cadherin is one of the principal members of the cadherin multigene family. It is a transmembrane glycoprotein that is a key component of epithelial cell adherence junctions and is involved in maintaining cellular and tissue integrity (22,33,41). Decreased expression of E-cadherin on epithelial cells may lead to cellular dedifferentiation and loss of cell:cell adherence. The cells become more mobile, more proliferative and are likely to undergo transformation, become invasive and eventually metastatic (8,42,61). This has been shown to be the case with prostate epithelial cells and decreases in E-cadherin have been correlated with high grade tumors (4,6,32,3738,53,54). E-cadherin has thus been considered to be a metastasis suppressor molecule and has been used as a molecular marker of metastasis and tumor recurrence (21,55). Other cadherins, such as neural (N-) and placental (P-) have also been shown to function in tumorigenesis (3,13,17,25,51). The presence of N-cadherin promotes binding of the tumor to the stroma, thereby facilitating invasion and metastasis. More precisely, however, it is the inverse expression of N-cadherin and E-cadherin that has been hypothesized to regulate the invasive and metastatic phenotype of the tumor (3,13,38,49).

A curative therapy for metastatic prostate cancer is non-existent. Cytokines are known for their cytostatic and immunomodulatory effects on many types of tumor cells (40). The modulation of adhesion molecule expression on cells, including tumor cells has been well documented (7). Sokoloff *et al.* examined the effect of cytokines on the expression of many prostate tumor markers, including ICAMs and cadherins (45). Their results showed that TNF-α upregulated ICAM-1, P-cadherin, but not E-cadherin on PC-3 tumor cells and that overall, cytokine treatment reduced binding of LNCaP and DU-145 but not PC-3 cells to human bone marrow stromal cells.

With regards to host cellular immune response to cancer, we have been investigating a non-classical effector cell, the eosinophil. Eosinophils have been most comprehensively studied for their role in parasitic infestations and in allergic hypersensitivity reactions, such as asthma, and they are implicated in the mucosal damage observed in bronchial asthma (5,11,36,43,45). Eosinophils, like lymphocytes, produce an array of cytokines, some of which: a) auto-regulate eosinophil growth, development and activation, b) modulate the immune response, c) exert direct anti-cancer activity on the tumor (24,51,60), and d) modulate adhesion molecule expression (24). They have also been found in many tumor infiltrates, however, their role as anticancer effectors has not been clearly defined (24,26,35). Although their presence has not been documented in prostate cancer histopathological preparations, their granular proteins have and thus we can deduce the prior presence of eosinophils (20). We have recently shown that activated eosinophils inhibit prostate tumor cell colony formation *in vitro* and that cytokines TNF-α and IL-4 are partially responsible for the cytotoxic activity against breast and prostate cells (20,27). In this study, the potential modulation of cell adhesion molecule expression on DU 145 and PC-3 prostate tumor cells was investigated.

MATERIALS AND METHODS

Eosinophils

Peripheral blood leukocytes (PBL) from eosinophilic allergic and asthmatic individuals were fractionated on a metrizamide density gradient (56). Hypodense (metrizamide fraction 22) and hyperdense (metrizamide fraction 24) eosinophilic fractions were collected, washed and counted.

Tumor cell lines

PC-3 and DU 145 are well characterized human tumor cells purchased from ATCC (American Type Culture Collection, Manassas, VA). Tumor cell lines were subcultured with their appropriate media, PC-3 with (F12K) containing sodium pyruvate, non-essential amino acids, gentamycin, penicillin and streptomycin and 7% fetal bovine serum and DU 145 with RPMI medium supplemented with sodium pyruvate, non-essential amino acids, gentamycin, penicillin and streptomycin and 10% fetal bovine serum.

24 hr Cultured supernatants

Eosinophils were isolated from peripheral blood of allergic individuals by metrizamide density gradient fractionation. The cells were incubated at a density of 1×10^6 cells/ml for 24 hr. The conditioned supernatants were collected and used to examine their effects on the expressions of CAM and cadherin on PC-3 and DU 145 prostate tumor cells.

Cytokines

TNF-α, IL-10 and IL-12 were purchased from R&D systems (Minneapolis, MN). Stock concentrations were prepared and stored at -30°C until ready for use.

Eosinophil: tumor cell coculture

DU 145 and PC-3 cells were seeded into T_{25} tissue culture flasks at a density of 5×10^5 cells/ml and the flasks were incubated at 37°C, under 5% CO_2 and 95% air culture condition for 24 hr. The media was removed and hyperdense eosinophils were added to the subconfluent flasks at 4: 1 E: T ratio for 30 min. The eosinophils were removed and the tumor cells were harvested, then examined for the presence of adhesion molecules by flow cytometry. In some experiments, 24 hr 6-well culture plates of DU 145 and PC-3 cells (seeded at 2.5×10^5 cells per well) were cultured with

eosinophils and examined microscopically at various times for binding of eosinophils to the tumor cells.

Antibodies

All antibodies were purchased from BD Biosciences, Pharmingen (San Diego, CA).

Analysis of adhesion molecule expression by flow cytometry

(ICAM-1, VCAM-1 and ELAM-1, N-cadherin, E-cadherin). Surface expression of adhesion molecules was determined (with and without treatment with eosinophils, eosinophil conditioned supernatants, IL-10 and IL-12) by flow cytometry (FACS Calibur, BD Biosciences, Mountain View, CA). Direct and indirect staining methods were used accordingly. In the direct assay, 1×10^6 cells were first washed with PBS containing 1% BSA (PBS/BSA, 1%), then incubated for 45 min with saturating amounts of FITC- or PE-conjugated mouse monoclonal antibody to detect the adhesion molecules. The cells were then washed with cold PBS/BSA (1%) and placed on ice until analysis. For indirect staining, cells were prepared as described for the direct assay. They were first incubated with primary antibody for 45 min, washed with cold PBS/BSA, 1%, then incubated for 30 min with goat anti-mouse IgG, (FITC- or PE-conjugated) before washing with cold PBS/BSA, 1%. For both direct and indirect assays, a total of 10,000 gated events were collected by the FACS Calibur cytometer. Cell Quest software (BD Biosciences) was used for analysis. The mean fluorescence intensity is reported.

RESULTS

Hypodense eosinophils bind to prostate tumor cells (Fig. 1)

In the hematoxylin and eosin stained 48 hr cocultures of hypodense eosinophils and PC-3 prostate cancer cells (5: 1 E:T Ratio), eosinophil clusters (arrows) can be seen bound to the tumor cells (Fig. 1B). The presence of eosinophils resulted in 80-90% death of the cells. This was not seen when eosinophils were cocultured with fibroblast cells (data not shown).

Hypodense eosinophils upregulate E-cadherin on DU 145 prostate tumor cells (Fig. 2)

Having demonstrated irreversible binding of eosinophils to prostate tumor cells, we then investigated which adhesion molecules were modulated by eosinophils during this event. DU 145 prostate cells did not express VCAM-1, ELAM-1 nor N-cadherin. Subculturing of DU 145 with eosinophils for 48 hr had no effect on VCAM-1 and ELAM-1, however, it resulted in N-cadherin expression and upregulation of E-cadherin.

The effect of eosinophil 24 hr conditioned supernatants on adhesion molecule expression on PC-3 tumor cells (Fig. 3)

Modulation of expression by conditioned supernatants from 24 hr cultures of hypodense eosinophils was also examined, along with cytokines IL-10 and IL-12. These cytokines, which have anticancer activity, can be produced by activated eosinophils. PC-3 cells did not express ELAM-1 and E-cadherin, but they did, however, show low level of expression of VCAM-1 and moderate levels of expression of N-cadherin. Eosinophil 24 hr conditioned supernatants were unable to induce E-cadherin expression. On the other hand, there was minor induction of ELAM-1 and N-cadherin. IL-10 up-regulated both VCAM-1 and N-cadherin, and induced the expression of ELAM-1 and E-cadherin. IL-12 also induced ELAM-1 and E-cadherin but failed to up-regulate VCAM-1 and down regulated N-cadherin.

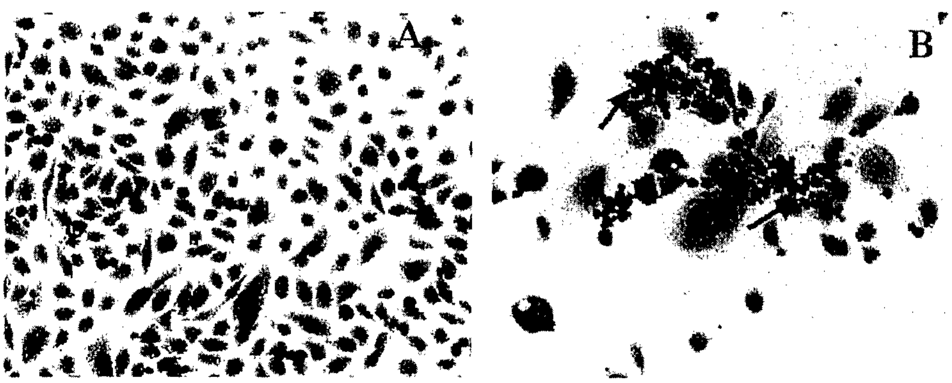

Fig. 1 Hypodense eosinophils bind to prostate tumor cells. Hypodense peripheral blood eosinophils were co-cultured (at 2: 1 E: T ratio) with a subconfluent monolayer of PC-3 prostate tumor cells (seeded in to 6-well culture plates at 2.5×10^5 cell/well 24 hr prior to the addition of eosinophils. The wells were observed microscopically at 2 hr, 24 hr and 48 hr. At 48 hr the wells were washed with PBS, methanol-fixed and stained with hematoxylin and eosin. Fig. A) shows PC-3 monolayer alone and in B) eosinophils can be seen bound (arrows) to the remaining tumor cells.

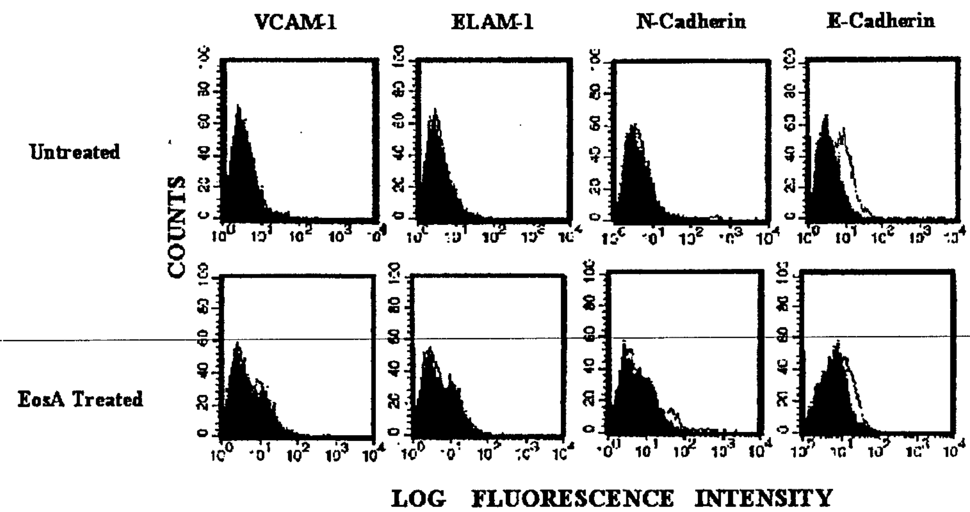

Fig. 2 Hypodense eosinophils upregulate E-cadherin on DU 145 prostate tumor cells. Peripheral blood hypodense eosinophils were cultured with DU 145 tumor cells (seeded into T_{25} culture flasks at 5×10^5 /ml 24 hr prior) at 4: 1 E: T ratio for 30 min. The tumor cells were harvested and examined by flow cytometry for VCAM-1, N-cadherin and E-cadherin expression.

Pro-inflammatory and non-inflammatory cytokines upregulate ICAM-1 on DU 145 and PC-3 tumor cells (Fig. 4)

Eosinophils produce both inflammatory and non-inflammatory cytokines, when activated. We selected the ICAM-1 adhesion molecule, which is constitutively expressed on most cells, including the two prostate cell lines, PC-3 and DU 145, to determine whether the two cytokines IL-10 and TNF-α, had opposite effects. TNF-α (inflammatory cytokine) up-regulated ICAM-1 on both PC-3 and DU 145 and IL-10 (non-inflammatory cytokine) upregulated its expression only on DU 145.

DISCUSSION

We hypothesized that activated eosinophils which may be found in tumor infiltrates produce cytokines which are both tumor enhancing and inhibitory. Moreover these cytokines may modify adhesion molecule expressions on tumor cells thereby modifying their motility and metastatic capabilities. Eosinophil coculture with DU 145 tumor cells had no effect on CAM expression, however, E-cadherin was significantly upregulated and N-cadherin was minimally expressed. PC-3 cells, on the other hand expressed N-cadherin, but not E-cadherin. This profile has been described as characterizing the most metastatic tumor phenotype, of which PC-3 is representative (3,13,38,51). Eosinophil 24 hr conditioned supernatants had little to no effect on this profile (N-cadherin was slightly upregulated). On the other hand, IL-10 which upregulated both VCAM-1 and ELAM-1, did alter this profile by upregulating E-cadherin and slightly upregulating N-cadherin. IL-12, however, significantly upregulated E-cadherin, but down regulated N-cadherin. TNF-α upregulated ICAM-1 on both DU 145 and PC-3. ICAM-1 is constitutively expressed on most cells, including tumor cells.

Although activated eosinophils produce cytokines which upregulate adhesion molecules, the failure of the 24 hr conditioned supernatants to modulate adhesion molecules could be due to the time (24 hr) of collection of the samples. There is heterogeneity in the kinetics of cytokine expression and a longer culture time may be required in order to capture a more comprehensive array of expressed cytokine proteins. The effect of kinetics of collection of eosinophil conditioned supernatants has been demonstrated against breast cancer cells *in vitro* (28). Another explanation for the failure of the eosinophil conditioned supernatants to affect CAM expression could be due to antagonism of cytokines and other expressed proteins (e.g. eosinophil granular proteins) thereby nullifying modulating effects on adhesion molecule expression. Studies are currently underway to examine collaborating effects, if any, between eosinophil expressed proteins on tumor cell growth. Both IL-10 and IL-12 have been well studied as anticancer cytokines. In mouse models, Stearns and Wang have demonstrated inhibition of tumor growth and metastasis by IL-10 (46). They have also shown inhibition of prostate tumor growth and metastasis in IL-10-transfected human prostate tumor lines

orthotopically implanted in a SCID mouse model (45). The mechanism by which IL-10 inhibited metastasis was by down-regulation of MMP-2 expression and up-regulation of TIMP-1 expression. IL-12, on the other hand upregulates NK and T cell activity which in turn exert anticancer activities (44,52,62). Hiscox's group, however, demonstrated inhibition of binding to human colon cancer cells to matrigel *in vitro* (18). Flow cytometry and immunohistochemistry analysis of IL-12-treated tumor cells revealed an up-regulation of E-cadherin (18). Our study with prostate cancer cells confirms this former observation that IL-12 up-regulates the E-cadherin metastasis suppressor molecule and offers yet another mechanism by which IL-10 can inhibit tumor metastasis. The potential collaborative action of an inflammatory cytokine (IL-12) and anti-inflammatory cytokine (IL-10) to suppress tumor metastasis is very intriguing, since they would be expected to act more in an antagonistic fashion. Although we have not examined this yet (i.e. combination IL-10/IL-12 treatment), we did find both cytokines in our EosAcs (to our knowledge the switch in cadherin profile on a highly metastatic, human anti-androgen resistant prostate tumor cell by cytokines has not been demonstrated before. Collection of supernatants at longer culture times (48, 72, 96 hr) will be examined on prostate tumor cell growth and adhesion molecule expression. These longer time periods may allow for greater release of cytokines at appropriate concentrations to affect a dramatic modulation of adhesion molecules on prostate tumor cells.

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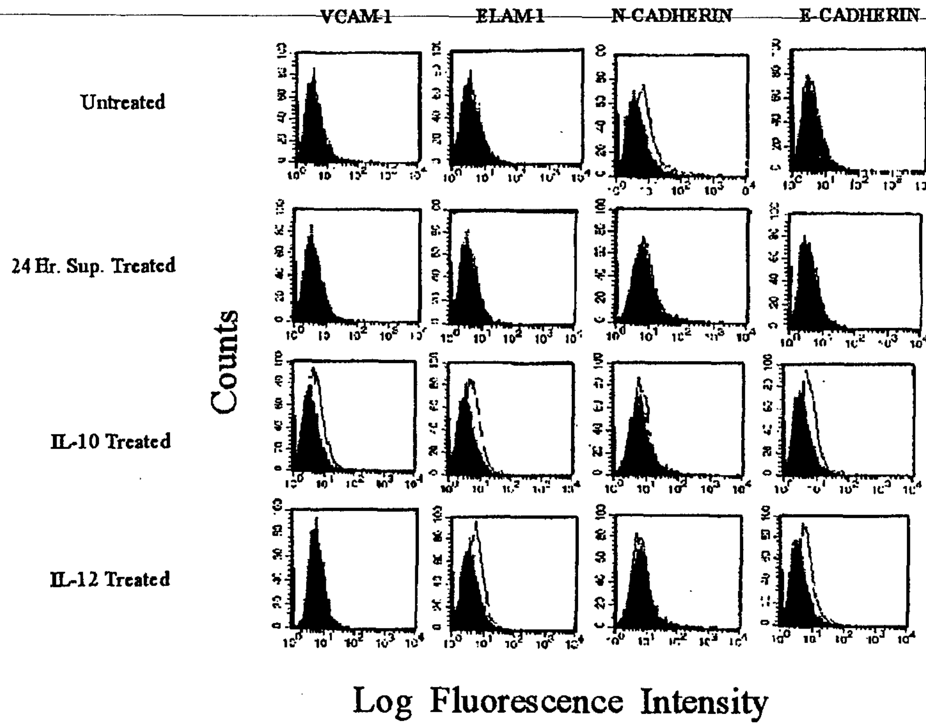

Fig. 3 The effect of eosinophil 24 hr conditioned supernatants on adhesion molecule expression on PC-3 tumor cells. T_{25} culture flasks were seeded as described in Fig. 2 with 5 x 10^5 tumor cells for 24 hr. The medium was removed and either eosinophil conditioned supernatant, IL-10 (1 ng/ml) or IL-12 (1 ng/ml) were added and the flasks incubated for an additional 24 hr. The tumor cells were trypsinized and harvested, then examined for adhesion molecule expression (VCAM-1, ELAM-1, N-cadherin and E-cadherin by flow cytometry.

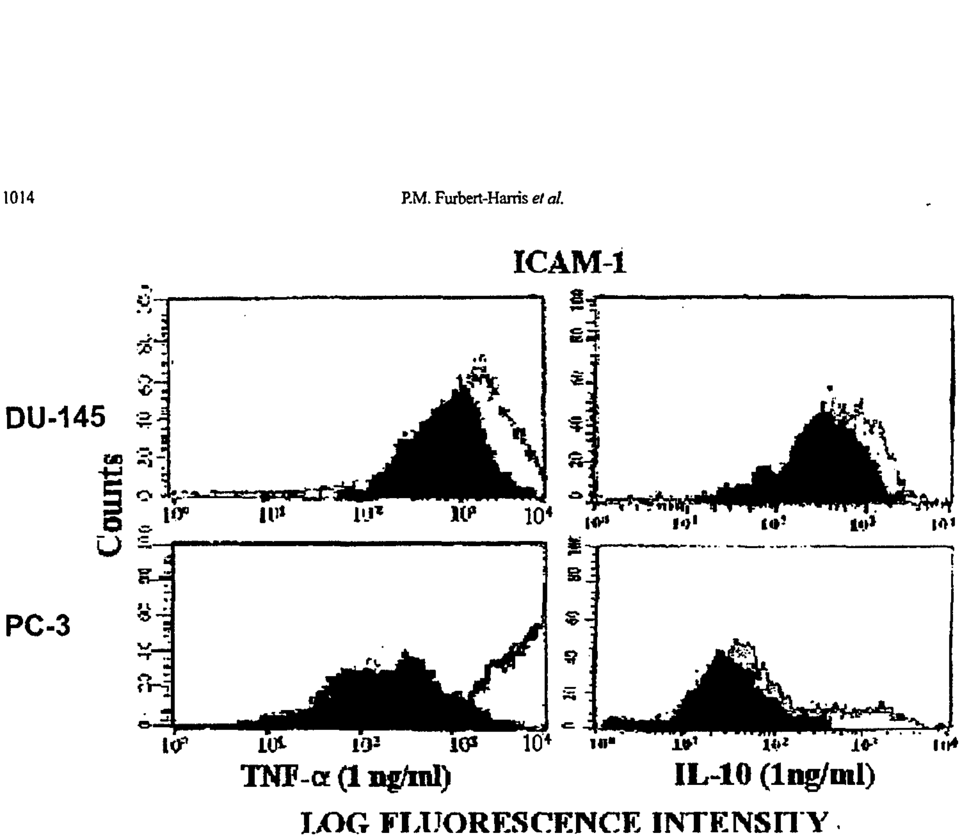

Fig. 4 Pro-inflammatory and anti-inflammatory cytokines upregulate ICAM-1 on DU 145 and PC-3 tumor cells. DU 145 and PC-3 tumor cells were treated with TNF-α and IL-10 as described in Fig. 3, then examined for ICAM-1 expression.

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EOSINOPHILS IN A TRI-CELL MULTICELLULAR TUMOR SPHEROID (MTS)/ENDOTHELIUM COMPLEX

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Abstract - Eosinophils have been found in infiltrates of many different cancers. It is still unclear as to whether they are passive bystanders in the cellular milieu or active cellular agents in host responses. Thus their harmful or helpful nature remains equivocal. We have developed an *in vitro* tri-cell model of eosinophils, MCF-7 breast tumor cell spheroids and HUVEC endothelial cells to examine the binding and association of eosinophils with both the tumor and the endothelia and the ensuing action of the tumor. Eosinophils bound very rapidly to the tumor spheroid and remained tightly bound throughout the 24 hr culture period. Histological staining of the tri-cell complex revealed highly granulated eosinophils as well as large amounts of degranulated protein diffused throughout the spheroid. IL-5 treatment of eosinophil: MTS complexes resulted in destruction of the tumor cells, particularly those which had grown out from the spheroid onto the endothelial cells. Eosinophils, pretreated with IL-5 before interaction with the tumor or endothelial cells, bound aggressively to the endothelial cells, thereby preventing tumor attachment. This eosinophil tri-cell tumor model system mimics clinical observations with regards to binding to epithelial and endothelial cells, dispersal of granular proteins throughout the tumor and also tumor destruction. Because it closely mirrors *in vivo* cellular interactions, it allows one to study more closely the mechanism(s) of eosinophil killing, the modulation of eosinophil activity and the testing of therapeutic interventions. The accommodation of the model to tumor invasion, using metastatic tumor cells and extracellular matrices such as matrigel, will help to elucidate a role for eosinophils (and their mediators) in cancer invasion and metastasis.

Key words: Eosinophils, multicellular tumor spheroids (MTS), interleukin-5

INTRODUCTION

Tumor metastasis is a complex multistep event which involves several discrete stages. Single tumor cells first invade the vascular or lymphatic circulation. This is followed by attachment to endothelial cells within target tissues, migration through the endothelium and underlying basement membrane, and finally proliferation within the target site (36). The initial step of tumor cell adherence to the vascular endothelium is a critical rate limiting step of the metastatic process and involves the association of complimentary cell adhesion and integrin surface molecules on tumor and endothelial cells (25,28). Cell adhesion molecules (CAM's) such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and endothelial leukocyte adhesion molecule (ELAM-1), all play a prominent role in normal cellular trafficking as well as during inflammatory responses when circulating leukocytes are recruited into tissue sites (26,29,31,45). Up-regulation of ICAM-1, VCAM-1 and ELAM-1 on endothelial is a prerequisite for leukocyte-endothelial cell adhesion, which must occur before extravasation (13,27). This adhesion is mediated by the binding of ICAM-1, VCAM-1 and ELAM-1 to specific integrin receptors, LFA-1, VLA-4 and Sialyl-Lewis X, respectively (1,5,19,21,40,41).

Tumor cells also express ICAM-1, VCAM-1 an E-LAM-1 (8,16,23,34,38,44,53) along with the integrins LFA-1, VLA-4 and Sialyl-Lewis X (2,11,22,32,39), and these molecules can be upregulated on both tumor and endothelial cells by various agents including inflammatory

Abbreviations: CAMs: cellular adhesion molecules; **ECGF**: endothelial cell growth factor; **ELAM-1**: endothelial leukocyte adhesion molecules-1; **HUVEC**: human umblical vein endothelial cell; **IL-1α**: interleukin-1 alpha; **IL-1β**: interleukin-1 beta; **IL-5**: interleukin-5; **LFA-1**: leukocyte function antigen-1; **MTS**: multicellular tumor spheroid; **VCAM-1**: vascular adhesion molecules-1; **VLA-4**: very late antigen-4

cytokines (18,32,38,40,48,49,53). Increased expression of ICAM-1, VCAM-1 and E-LAM-1 is correlated with invasiveness and metastasis (46,53). Although cytokines IL-1α, IL-1β and TNFα are cytostatic/cytotoxic to tumor cells, they also upregulate adhesion molecule expression on both endothelial and tumor cells (16,44).

Eosinophils are granulocytic leukocytes which play a key role in helminthic infections as well as allergic inflammatory reactions (15,37,50). They have been found in inflammatory infiltrates of various cancers, including breast (42). We have previously shown that activated eosinophils have both cytostatic and cytotoxic effects on tumor cells *in vitro*, and moreover when tumor cells grow in multicellular spheroids, eosinophils can bind and infiltrate the spheroid (14). Eosinophils, upon activation, produce a host of mediators; including inflammatory and non-inflammatory cytokines which can a) modulate eosinophil activity; b) modulate adhesion molecule expression on endothelial cells, and c) exert direct growth inhibition of the tumor cells (42). In this investigation we are developing an *in vitro* tri-cell tumor model system in order to study the impact of eosinophil infiltration of the tumor on its binding to endothelial cells as a first step in preventing invasion and metastasis. In this present report we have used the non-invasive breast tumor cell MCF-7, the human umbilical vascular endothelial cell (HUVEC) and eosinophils to determine fitness of the model.

MATERIAL AND METHODS

Cell lines

– *MCF-7* breast tumor cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA). The cells were propagated in RPMI medium supplemented with sodium pyruvate, non-essential amino acids, gentamycin, penicillin/streptomycin and 10% fetal bovine serum (complete medium).

– *Eosinophilic cell lines* (developed in our laboratory with peripheral blood eosinophils from allergic individuals) were cultured and maintained in RPMI complete medium.

– *HUVEC* endothelial cells were also obtained from ATCC, and cultured in Ham's F-12K medium with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate and supplemented with 0.1 mg/ml heparin and 0.3 mg/ml endothelial cell growth factor (ECGF), and 10% fetal bovine serum, gentamycin (50 µg/ml) and penicillin/streptomycin (100 units/ml, 100 µg/ml, respectively).

Multicellular tumor spheroids

Tumor cells were dispensed into non-vented T_{25} tissue culture flasks (1×10^6 cells/flask) to which 10 ml RPMI complete medium was added. The flasks (loose-capped) were placed in a 37°C incubator, under 5% CO_2 95% O_2 conditions for 5-10 min, capped, removed and placed in a 37°C incubator (without CO_2) on a rocking platform for 24-48 hr (rocking at 30 revs/min). The tumor aggregates [multicellular tumor spheroids (MTS)] were placed in 100 mm Petri dishes or 6-well plates containing 0.5% agarose overlay. Fresh medium was added and changed every two days until assay use.

Eosinophils: MTS: HUVEC tri-cell complex

Seven days old MCF-7 MTS were cultured in 6-well tissue culture plates overnight with medium alone, hypodense eosinophils (10,000: 1 and 1000: 1 E: T ratios) pretreated with IL-5 (5 ng/ml) or with IL-5 present in the culture medium. MTS with associated eosinophils were first transferred to 6-well plates containing medium to remove unbound eosinophils. Eosinophil: MTS were then transferred to 6-well tissue culture plates, containing a confluent lawn of HUVECs (each well seeded with 3×10^5 cells 48 hr before use). The plates were cultured at 37°C for 1, 4 and 24 hr. Each treatment (medium alone, IL-5 pretreated eosinophils and eosinophil: MTS with IL-5, in culture 24 hr prior to) and each culture time was set up in duplicate. After incubation, the medium was removed, the wells were washed with PBS then stained with Giemsa May-Grunwald. The slides and culture wells were examined microscopically using an Olympus BX60 phase contrast microscope with video. Data were captured on computer files using Optronics software.

RESULTS

Eosinophils bind to MCF-7 monolayers and multicellular spheroids

Within 30 min to 1 hr, eosinophils bound to both the MCF-7 monolayer (Fig. 1A) and to the MTS (Fig. 1B, 1C). Fig. 1B depicts a spheroid (600 µm in size) with eosinophils bound (solid arrow) and unbound (dashed arrow), and Fig. 1C shows bound eosinophils to a spheroid with necrotic core. Binding was irreversible.

Multicellular tumor spheroids bind to HUVEC endothelial cells

Within 1 hr (Fig. 2A) MCF-7 MTS bound to the HUVEC monolayer. The tumor cells spread from the MTS out over the endothelial lawn by 24 hr (Fig. 2B) and by 48 hr (Fig. 2C) the integrity of the endothelial lawn was lost.

Eosinophil: MTS: HUVEC: complex (1 hr culture time)

When 24 hr eosinophil: MTS complexes were added to the endothelial lawn, MTS bound to the HUVEC lawn as early as 1 hr. Eosinophils can be seen bound to the MCF-7 spheroid which itself is attached to the HUVEC monolayer (Fig. 3B). The arrow is pointing to a single bound eosinophil in Fig. 3B and 3C at 20x and 100x magnification, respectively. When the eosinophils and tumor spheroid were treated with IL-5 (5 ng/ml) for 24 hr then added to the endothelial monolayer, (Fig. 3D and 3E), unlike the non-IL-5 treated (Fig. 3G) the entire spheroid was covered with eosinophils, and eosinophil clusters (arrow) can be seen binding to the HUVEC monolayer. At higher magnification eosinophil granules can be seen throughout the spheroid (Fig. 3E, see arrow). On the otherhand, when the eosinophils were pretreated with IL-5, then added to the MCF-7 MTS on the HUVEC lawn of cells, the eosinophils bound rapidly to the HUVECs (Fig. 3F), preventing the tumor spheroids from binding.

Eosinophil tri-cell complex (24 hr culture time)

Tri-cell complexes, with and without IL-5 treatment,

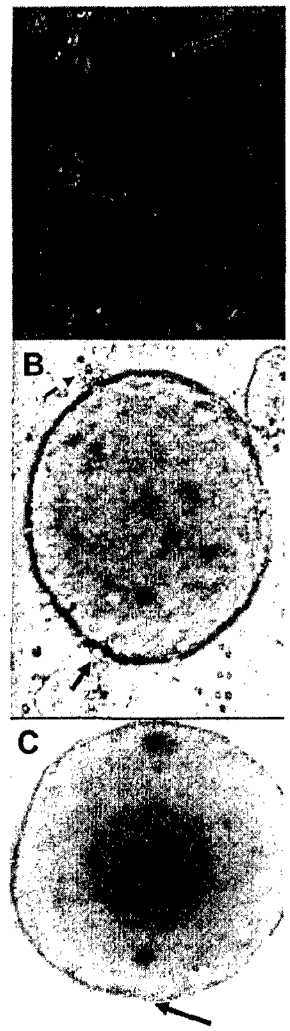

Fig. 1 Eosinophils bind to MCF-7 monolayers and MCF-7 multicellular spheroids. MCF-7 tumor cells were seeded into 6-well cluster plates at 3 x 10^5 cells /well. The plates were then incubated for 48 hr. Fresh medium was added, along with eosinophils at an E:T ratio of 4: 1 (A). Eosinophils were also cultured with 7-day old MTS in 6-well plates with agarose overlay at E:T ratio of 1000: 1 for 24 hr (B and C). Both plates were observed and photographed every 30 min for the first two hours, then at the end of the assay. The media was removed, the cells fixed and stained with hematoxylin and eosin. Magn. **A**, **B**) x 20

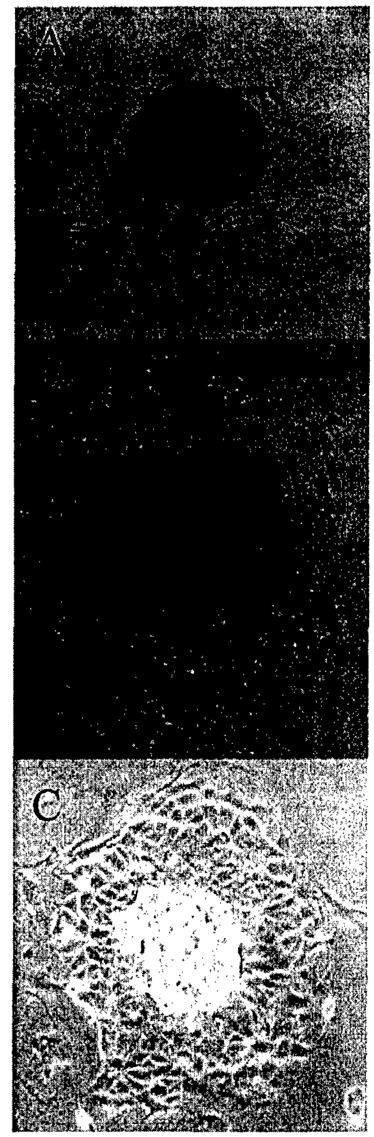

Fig. 2 Multicellular tumor spheroids bind to HUVEC endothelial cells. Seven days old MCF-7 MTS were added to confluent monolayers of HUVEC cells. The cultures were incubated for 48 hr. Photomicrographs were taken every 30 min for the first 2 hr (**A**), then at 24 (**B**) and 48 hr (**C**). Magn. **A**) x 10; **B**) x 20; **C**) x 40

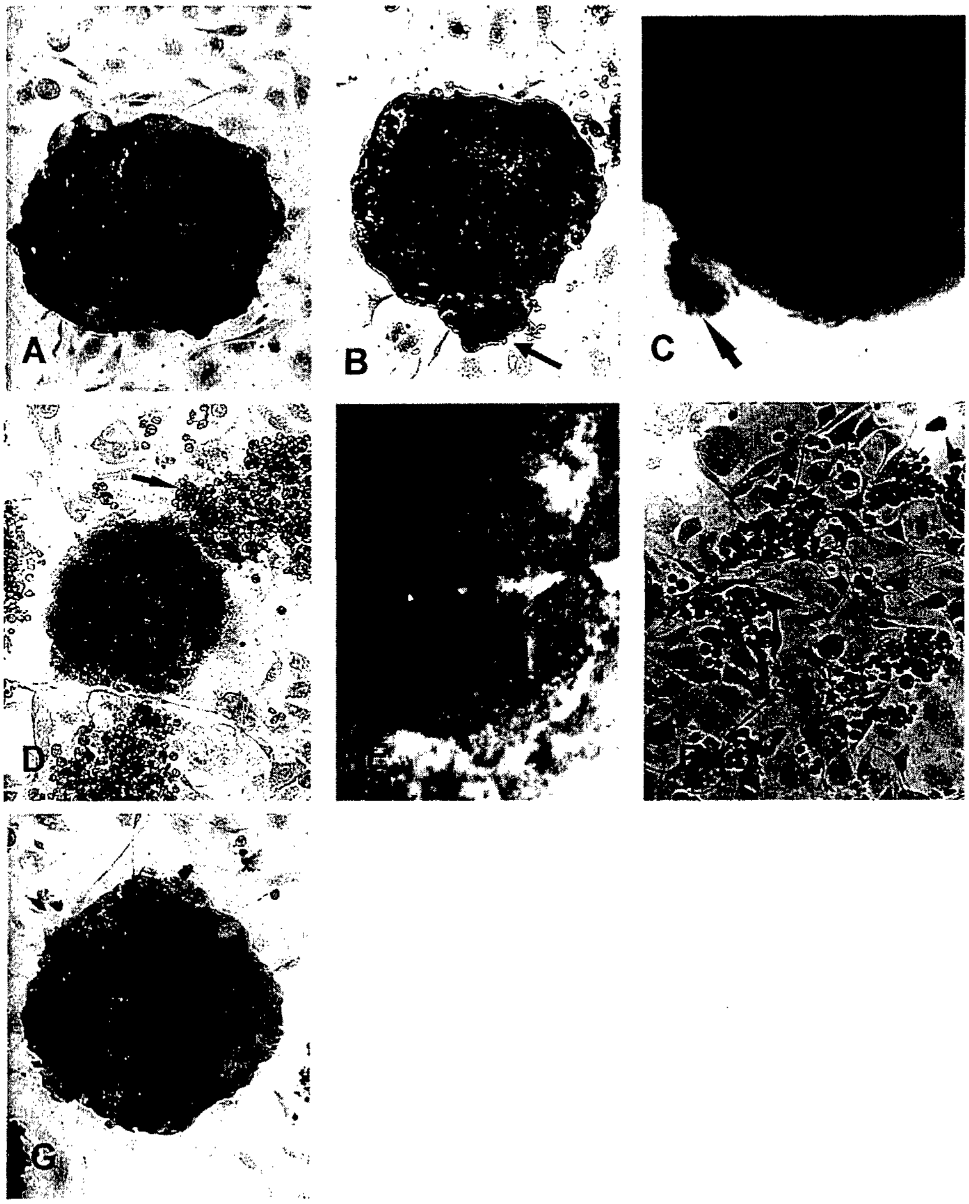

Fig. 3 MCF-7 MTS were cultured on HUVEC monolayer for 1 hr (**A**). 24 hr Eosinophil:MTS complexes were cultured on HUVEC cells for 1 hr (**B** and **C**). Eosinophils and MCF-7 MTS were cultured with IL-5 at 5 ng/ml (**D** and **E**) for 24 hr, then added to a monolayer of HUVEC cells for 1 hr. IL-5 untreated (**G**) and pre-treated (**F**) eosinophils were also added to MTS and HUVEC cells for 1 hr. All samples were fixed and stained with Giemsa/MayGrunwald. Magn. **A**, **B**, **D**, **F**, **G**) x 20; **C**, **E**) x 100

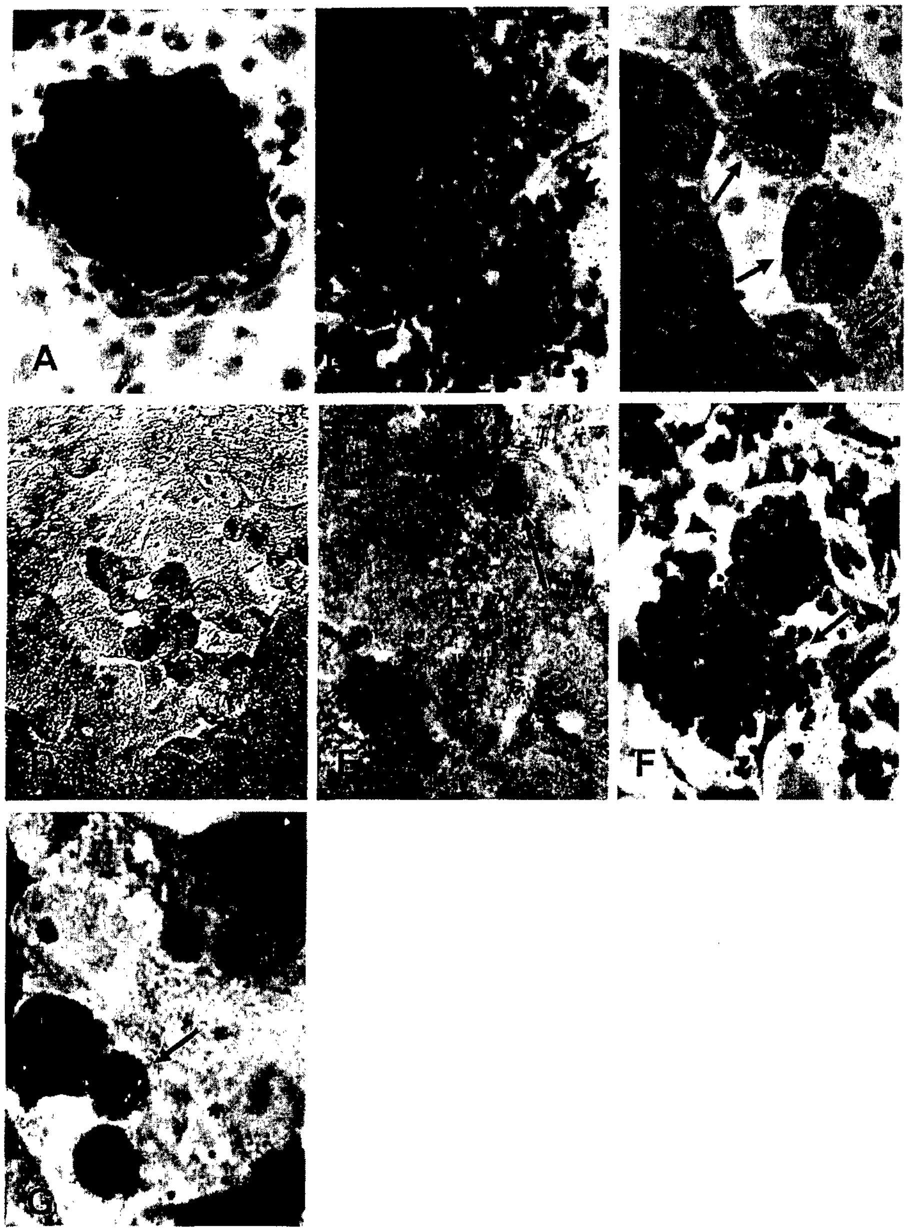

Fig. 4 Eosinophil tri-cell complex (24 hr culture time). Eosinophils were cultured and all treatments were similar to that described in Fig. 3. The tri-cell complex, however, was incubated for 24 hr. **A)** control MTS on HUVECs; **B)** and **C)** eosinophil: MTS + HUVEC; **D)** and E) eosinophil: MTS + IL-5 (24 hr) + HUVEC (24 hr); **F, G)** eosinophils + IL-5 (24 hr) + MTS and HUVEC (24 hr). Magn. **A, D)** x 40; **B, F)** x 20; **C, E, G)** x 100

were cultured for 24 hr. In the untreated group, MCF-7 spheroids were spread out along the HUVEC monolayer (Fig. 4A). The presence of eosinophils, bound and infiltrated, (Fig. 4B) did not affect the spread of the tumor cells. Individual eosinophils (arrows) can be seen more readily at higher magnification (Fig. 4C) bound to tumor cells. In Fig. 4D (40x magnification), tumor cells are destroyed and there are many apoptotic-like bodies throughout, along with high numbers of eosinophils and granular protein. In Fig. 4E, the arrows point to eosinophils within the spheroids (100x magnification). 24 hr tri-cell complexes with IL-5 pretreated eosinophils were similar to the 1 hr cultures in that the eosinophils bound vigorously to the endothelial cells (Fig. 4F), prohibiting the binding of the tumor. In Fig. 4G eosinophils are seen attached to the HUVEC cells (100x magnification).

DISCUSSION

The presence of eosinophils in and around human tumors has been well documented (6,17,20,30,33,42,52). However, their role and mechanism(s) of action are yet to be delineated. The current study represents the initial stages in the development of an eosinophil: tumor model system which can be used to examine eosinophil activity and modulation of that activity by various mediators. Moreover, the relationship of the individual cellular participants can be more intimately examined. The rapid and constitutive binding to and infiltration of the tumor spheroid mimics that reported in clinical studies (7,43) and the deposition of granular proteins throughout also mirrors that described *in vivo* (20,42,43).

Untreated eosinophils bound rapidly and tightly to the tumor spheroid (within 1 hr). This early time frame was arbitrarily chosen, but studies by Taylor *et al.* (47) have demonstrated binding of eosinophils to tumor cells as early as 15 min. Both tumor cells and eosinophils bind to the endothelia by way of cell adhesion molecules and integrins (3,12,51). IL-5 treatment of the eosinophils and MTS, and of the eosinophils alone markedly upregulated eosinophil binding to both the tumor spheroid and to the endothelium, in fact pretreatment of eosinophils resulted in rapid binding to the endothelial cells, thereby preventing the tumor spheroids from binding. IL-5, has been demonstrated by others to upregulate eosinophil adhesion molecules (9,54). This could be one of the reasons why the eosinophils were able to bind so effectively. In contrast, eosinophils without IL-5 treatment also bound very rapidly to the tumor and this implies that either the CAM molecules upregulated by IL-5 (e.g. I-CAM-1, V-CAM) may be constitutively expressed by eosinophils, tumor and endothelial cells, or that there are other adhesion molecules involved. We and others have demonstrated such constitutive presence of these CAMS (3). In this model we deduce that the eosinophils have been activated because the granular proteins are seen throughout the spheroids (Fig. 3, 4). On the other hand, we also observed granular proteins in the spheroids that were infiltrated by non-IL-5 treated eosinophils (Fig. 3). This suggests that perhaps the tumor itself is releasing something which is activating the eosinophils to degranulate. This has been described by Ali *et al.* (3) who demonstrated that TNF-α stimulated breast carcinoma cells express mediators that can bind and activate eosinophils and that anti-Rantes antibody introduced into breast carcinoma cell supernatants partially blocked eosinophil activation. After 24 hr co-culture of the tri-cell complex, having been treated with IL-5 (Fig. 4D), there is extreme damage to the tumor cells, as well as dense areas of eosinophils and granular protein. The presence of apoptotic-like bodies suggests that one mechanism of eosinophil killing could be by apoptosis induction. Eosinophils produce granzyme B and perforins, proteins which are the hallmark of cytotoxic T cell (CTL) and natural killer (NK) tumor killing (4,35). This mechanism of killing for eosinophils has been studied by Costain et al in a murine tumor model (10). Studies are ongoing to determine whether the observed cell death is apoptosis or some other form of programmed cell death such as autophagy as described by Kanzawa *et al.* in malignant glioma cells treated with arsenic trioxide (24).

We have started the initial stages of this eosinophil: tumor: endothelium tri-cell model with which we can now probe with questions about each of the cells. We can also modulate the activity of each cell and study effects. Modifications of the model to utilize metastatic tumor cells in an invasion assay will allow us to assess the influence of eosinophils on invasion and metastasis. Future evaluation of this model by confocal microscopy and electron microscopy will more definitively delineate the cellular interactions, and molecular examination of specific markers will aid in determining a clearly defined role of eosinophils as anti-cancer effector cells.

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Inhibition of Prostate Cancer Cell Growth by Activated Eosinophils

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BACKGROUND. Host Immune response to prostate cancer primarily involves the CTL and NK effector cells. Recent immunotherapeutic strategies incorporating cytokine genes into the tumor cell and/or dendritic cells have had encouraging results. In this study, we describe the inhibitory activity of a third potential effector cell, the eosinophil, against DU 145 and PC-3 prostate tumor cells growth in vitro.

METHODS. Subconfluent monolayer cultures of DU 145 and PC-3 cells were incubated with peripheral blood eosinophils from allergic or asthmatic individuals and also with eosinophil cultured supernatants. Newly established eosinophil cell lines were also studied. After harvesting, the plates were washed and stained with Hematoxylin/eosin (H/E) then photographed. The combination of monolayer cell growth inhibition and colony formation inhibition assays were used to evaluate eosinophil inhibitory activity. In the colony formation inhibition assay one hundred cells per well in 6-well plates were incubated overnight, after which peripheral blood eosinophils, conditioned media and cytokines, IL-4 and TNF-α were added. The plates were harvested after 10 days incubation period. Colonies were stained and counted.

RESULTS. Hypo- and hyperdense peripheral blood eosinophils from allergic and asthmatic individuals as well as eosinophil cell lines established from these subpopulations inhibited both DU 145 and PC-3 cell growth at 58–78% and 10–38%, respectively. IL-5 up-regulated eosinophil cell line activity by 21–24%. The conditioned media which contained the released mediators of activated eosinophils were potent in their actions on both DU 145 and PC-3, inhibiting colony formation by as much as 90–100%.

CONCLUSION. These results clearly demonstrate the inhibitory potential of activated eosinophils and their released "soup" of mediators and therefore support the hypothesis that eosinophils may participate in host response to prostate cancer together with CTLs and NK cells. Furthermore, this study offers insights into possible strategies for enhancing eosinophilic activity in prostate cancer. *Prostate 57: 165–175, 2003.* © 2003 Wiley-Liss, Inc.

KEY WORDS: **eosinophils; prostate cancer; cytokine; DU 145; PC-3; cytotoxic T lymphocytes (CTLs)**

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INTRODUCTION

There are virtually few, if any, treatment options for hormone resistant, advanced stage prostate cancer, unlike early stage local cancer which can be very successfully treated [1,2]. The failures of most immunotherapeutic strategies in the past have been due to: (a) lack of tumor specific antigens which resulted in high levels of nonspecific immunity and (b) weakly immunogenic tumor specific antigens which give rise to less than adequate immune responses [2]. The use of cytokines (e.g., IL-2) in the past have rendered moderate success because of ineffective presentation to the effector T cells at the tumor site [3]. However recent studies in cancer immunotherapy have shown that dendritic cells (DCs) were more efficient as antigen presenting cells (APCs) and were the only APCs that could prime naïve T cells and induce them to mature into antigen-specific effector cells [4,5]. As a result of this, investigators have consequently transfected DCs with RNA-encoding prostate-specific antigen (PSA) and with amplified RNA from whole tumor to induce prostate specific cytotoxic T lymphocytes (CTLs) responses *in vitro* [6–11]. The resulting polyclonal responses which included anti-PSA responses as well as those to yet unknown tumor specific antigens, were broader and more effective in their activity. Pruitt et al. [11] demonstrated that the response to PSA was the dominant one, even though there was some level of activity against self-antigens present on benign prostate tissue. These types of studies are currently being used in clinical trials [12,13].

The natural killer (NK) cell had also received much attention because of its ability to selectively kill tumor cells and because of IL-2 induced augmentation of its tumoricidal activity [14,15]. Recent studies have demonstrated a strong correlation between NK activity and prostate cancer spread as well as tumor grade. The use of NK activity as a probe for tumor metastasis has proven useful and statistically reliable similarly to PSA serum levels [16,17]. Studies on NK activity in prostate cancer involving gene therapy with cytokines and other therapeutic agents have strongly delineated the role of NK cell activity [18–22].

In the present study, we have examined the role of a third "super" effector cell in cancer immunotherapy. Eosinophils are highly recognized as inflammatory cells in helminthic infections, and allergic asthmatic reactions, but there is a controversy about their role in cancer. Eosinophil infiltrates have been readily detected in histological preparations of tumor tissues from various tumor types (e.g., breast, cervical, colon, and lung), but none currently in prostate cancer [23–29]. Nonetheless, eosinophil crystalloids have been detected at prostate tissue sites and their presence has been inversely correlated with Gleason grade [30]. The fact that combination of eosinophil granular proteins (e.g., MBP) and cytokines (e.g., IL-4, IL-12, TNF-α) have anticancer activities [19,31–34] and most significantly, eosinophil perforins and granzyme B may mediate apoptotic destruction of tumor cells similarly to CTLs and NK cells [35,36] led us to investigate whether activated eosinophils and IL-5 treated eosinophil cell lines could inhibit prostate tumor cell growth in vitro.

MATERIALS AND METHODS

Peripheral Blood Eosinophils

Buffy coats of dextran-sedimented peripheral blood leukocytes (PBLs) from allergic and asthmatic individuals were washed and layered onto metrizamide density gradients with refractive indexes of 1.3620, 1.3650, 1.3665, 1.3680, 1.3695, and 1.3710 which corresponded to metrizamide percentages of 18, 20, 21, 22, 23, and 24, respectively [37]. These samples were centrifuged at 2,500 rpm for 30 min. Fractions 21 and 22 contained hypodense eosinophils while fractions 23 and 24 contained hyperdense eosinophils. Instead of pooling fractions, we selected 22 (hypodense) and 24 (hyperdense) fractions for this study. These fractions were chosen because of reports by others [38,39] and subsequent confirmation by us that they contain high percentages 85–90% of hypodense M22 and hyperdense M24 eosinophils.

Eosinophilic Cell Lines

The eosinophil cell lines GRC.014.22 and GRC.014.24 were established in our laboratory by transforming the metrizamide density gradient fractions 22 and 24, obtained above, with Epstein-Barr virus.

Eosinophil Sublines

These cell lines were obtained by FACS sorting of parental lines, GRC.014.22 and GRC.014.24 with monoclonal antibody, anti-CCR3, yielding sublines GRC.014.22S and GRC.014.24S, respectively. Further sorting of $CCR3^+$ cells (GRC.014.24S) was done using monoclonal antibody anti-CD49d to obtain GRC.014.24S.CD49d cells. The eosinophil parental cell lines and their sublines were routinely treated with mycoplasma removal agent and they tested negative for mycoplasma.

Conditioned Media (Supernatants)

Peripheral blood eosinophils (1.0×10^6 cells/ml) were cultured for 24 hr in complete RPMI 1640 media [supplemented with penicillin/streptomycin (100 U/ml/100 μg/ml), gentamycin (50 μg/ml), and 10% fetal bovine serum (FBS)]. Replicates of eosinophil parental

cell lines and sub-lines (1.0×10^6 cells/ml) were cultured in complete RPMI 1640 media for 24, 48, and 72 hr. Supernatants were collected after centrifugation at 1,500 rpm for 30 min, aliquoted and stored at −80°C until ready for use.

Tumor Cell Lines

DU 145 was purchased from American Type Culture Collection (ATCC), Manassas, VA. It was propagated in complete RPMI 1640 media in a humidified atmosphere of 5% CO_2 and 95% air. The PC-3 tumor cell line was also purchased from ATCC. This cell line was propagated in Hank's F12K medium (ATCC), [supplemented with penicillin/streptomycin (100 U/ml/100 μg/ml), gentamycin (50 μg/ml), and 10% FBS]. Both cell lines grow in culture as monolayers, and were routinely treated with mycoplasma removal agent and tested for mycoplasma. All cell cultures tested negative for mycoplasma.

EXPERIMENTAL METHOD

Inhibition of tumor cell growth was the standard end-point in this study. This was determined by monolayer cell growth inhibition and colony formation inhibition assays. Growth inhibition of DU 145 and PC-3 monolayer cells and colonies were assayed with activated eosinophils and with conditioned media from activated eosinophils and eosinophil cell lines.

Monolayer Growth Inhibition

Eosinophil: tumor. Tumor cells were seeded into either 6-well cluster plates at 3×10^5 cells/well or 12-well cluster plates at 1.5×10^5 cells/well. The plates were incubated for 24 hr at 37°C in an atmosphere of 5% CO_2 and 95% air. The media were removed at the end of incubation. Eosinophils from peripheral blood of allergic and asthmatic individuals and from eosinophil cell lines were added to each well at various effector:-target (E:T) ratios, and the plates were further incubated for 48–72 hr, the time at which control wells (media alone) became confluent. Replicates of test and control wells were carried out in all the experiments.

Conditioned media and cytokine inhibition. The effect of conditioned media and cytokines on tumor cell growth was determined similarly as described above. After 24 hr of initial incubation, the cell media were removed and the conditioned media or cytokines, IL-4 and TNF-α were added at various concentrations. The plates were further incubated for 48 and 72 hr as described above. Fresh media were added to replicate wells, which served as controls. At the end of the incubation period, the cells were harvested, washed 3× with PBS, fixed and then stained with H/E. The data were qualitatively analyzed by means of light microscopy and photodocumentation. The data were also quantified by direct measurement of cell density per well using a Chemi-imager 4000 (Alpha Innotech Corp., San Leandro, CA) analysis, which is briefly described as follows. First, the well area was determined and saved as the spot overlay. This was used throughout to overlay each well in the plate in order to standardize the area of all the wells. Then wells were selected, and their integrated density value (IDV) was determined. The IDV is the sum of all pixel values after background correction. Because the average value (AVG) is equal to the IDV per area (whereby area is constant), we used AVG as a comparative value. We then compared the IDV of the control (media alone) with that of the test samples. The percent inhibition was calculated as follows:

$$\%\,\text{Growth inhibition} = \left[\frac{\text{Average IDV (control)} - \text{Average IDV (test)} \times 100}{\text{Average IDV control}}\right]$$

Colony Formation Inhibition Assay

Six-well culture plates were seeded with 10^2 tumor cells/well. The plates were incubated overnight to allow the cells to adhere, after which the media were removed and peripheral blood eosinophils (with and without IL-5) were added at various E:T ratios in replicates. The plates were then incubated for 10 days. Control wells contained media alone. At the end of the incubation period, the effector cells were removed, the wells were washed 3× with PBS, fixed with methanol and then stained with HE. The colonies were counted manually for both test and control in a double blind fashion. Conditioned media from both peripheral blood eosinophils, eosinophil cell lines and also cytokines IL-4 and TNF-α were assayed similarly for inhibition of colony formation. Percent inhibition was determined as follows:

$$\%\,\text{Inhibition of colony formation} = \left[\frac{\text{Average no. of colonies (control)} - \text{Average no. of colonies (test)} \times 100}{\text{Average no. of colonies (control)}}\right]$$

ELISA Analysis

The ELISA assay was used to measure cytokine concentrations in conditioned media from cultured peripheral blood eosinophils (hypo- and hyperdense) and eosinophil cell lines. ELISA kits for IL-4, TNF-α, and others were purchased from R&D Systems (Minneapolis, MN). These assays were carried out according to manufacturer's procedure.

RESULTS

Peripheral Blood Eosinophils Inhibit PC-3 Tumor Cell Growth

In Figure 1A, hypodense eosinophils obtained from the peripheral blood of an allergic individual inhibited PC-3 tumor cell growth in a dose-dependent fashion. Inhibition ranged from 58% at E:T ratio of 5:1 to 75% at 25:1, while inhibition by the hyperdense fraction was even greater at both 5:1 (78%) and 10:1 (75%). These growth inhibitions were statistically significant at $P < 0.001$. Growth inhibition observed with tumor cell monolayers was confirmed by colony formation inhibition assay as shown in Figure 1B. Both hypo- and hyperdense eosinophils inhibited colony formation in a dose-dependent manner. Percent inhibition was comparable, ranging from 52% at E:T ratio of 5:1 to 88% at 50:1 (hypodense) and 66% at 5:1 to 92% (hyperdense), both with P values <0.0001.

Growth Inhibition of DU145 and PC-3 by Peripheral Blood Eosinophils From Non-Allergic Individuals

In Figure 2, nonallergic peripheral blood eosinophils were less effective in inhibiting tumor cell growth. Inhibition of PC-3 monolayer growth (Fig. 2A) ranged from 22% (1:1) to 36% (10:1). The activity against DU 145 was 12% at 1:1 and decreased to zero at 10:1. Inhibition

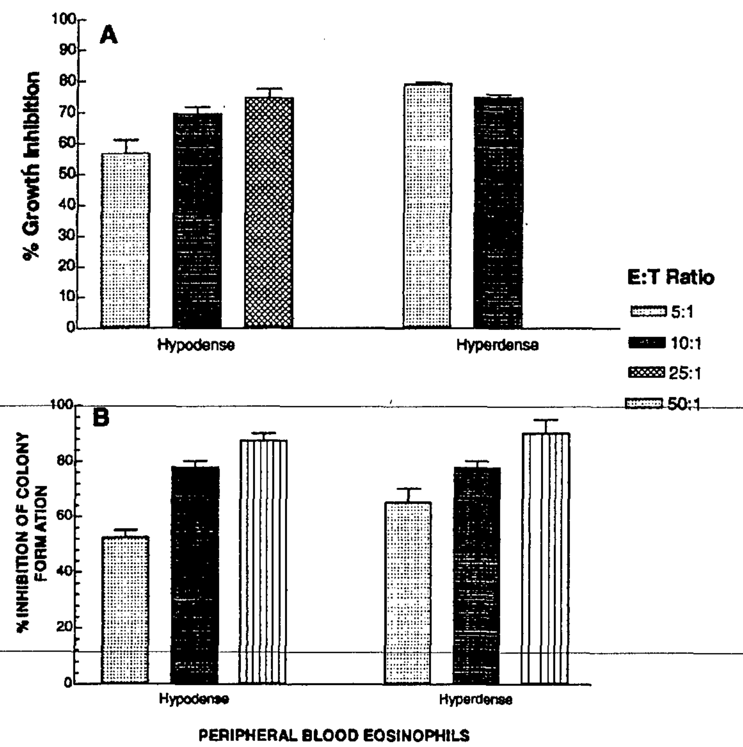

Fig. 1. Peripheral blood eosinophils inhibit PC-3 tumor cell growth. **A**: PC-3 tumor cells were seeded in replicates into 12-well plates at 1.5×10^5 cells/well and incubated overnight. Eosinophil subpopulations (hypo- and hyperdense) were added at various E:T ratios (5:1, 10:1, 25:1). The plates were incubated for an additional 72 hr, rinsed 3× with PBS, fixed, stained, and the percentage of growth inhibition determined. **B**: PC-3 tumor cells were seeded in replicates at 10^2 cells per well. After 24 hr incubation, hypo- and hyperdense eosinophils were added at various E:T ratios (5:1, 10:1, 50:1). The plates were incubated for an additional 10 days. The number of colonies was enumerated.

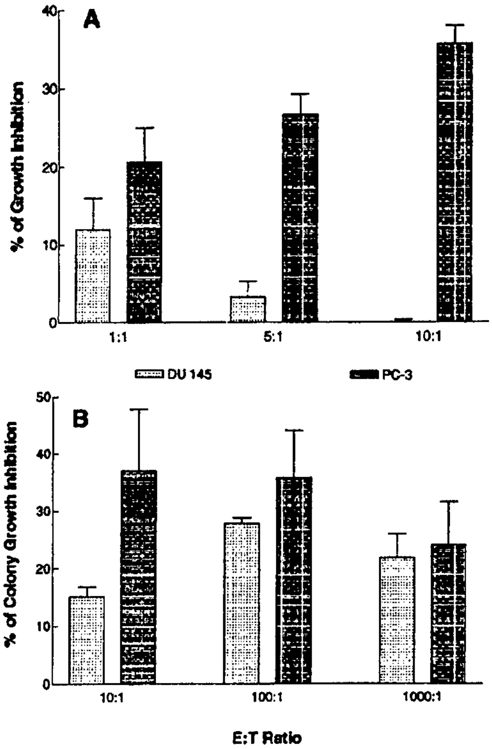

Fig. 2. Growth inhibition of DU145 and PC-3 by peripheral blood eosinophils from nonallergic individuals. DU145 and PC-3 tumor cells were seeded in 12-well culture plates similarly to that described in Figure 1, i.e., at 1.5×10^5 cells/well and incubated overnight. Pooled eosinophils fractions (hypo- and hyper) were added at E:T ratios 1:1, 5:1, and 10:1. (because of the low numbers of eosinophils obtained, we pooled the normo- and hyperdense fractions). The plates were incubated for 72 hr, rinsed with PBS, fixed and stained. Test and control wells were performed in triplicates. The percent growth inhibition was determined. Experiments were set up in triplicate. Colony growth innibition assays, DU145 and PC-3 tumor cells were seeded at 10^2 cells per well. Post 24 hr incubation, eosinophils fractions were added at 10:1, 100:1, and 1000:1 E:T ratios. The plates were incubated for 10 days at 37C, 5% CO_2. The number of colonies were enumerated.

of PC-3 colony growth confirmed that seen with the monolayer assay. PC-3 was more sensitive than DU 145 tumor cells.

Inhibition of Prostate Tumor Cell Growth by 24 hr (Eosinophil Conditioned Media)

In an effort to determine whether inhibition was mediated by soluble substances released by eosinophils, tumor cells were treated with 24 hr. conditioned media collected from hypodense and hyperdense eosinophil fractions from several allergic individuals. As summarized in Figure 3A, the eosinophil conditioned media of hypo- and hyperdense fractions from all donors significantly inhibited growth of DU 145 and PC-3 tumor cells by 22–68% with P values <0.05 to <0.0001. The conditioned media from donor 1 hypodense eosinophils had minimal growth inhibitory effect on DU 145 and PC-3 tumor cells (26% and 22%, respectively). The conditioned media from donor 3 hypodense eosinophils were more effective in inhibiting DU 145 cell growth than hyperdense eosinophils from the same donor (52% vs. 22%), while the conditioned media from donor 4 hyperdense eosinophils were more potent against the growth of both tumor cell lines. However, the percent growth inhibitions of DU 145 and PC-3 tumor cells by conditioned media from donor 4 hyperdense eosinophils were similar to those of donor 3 hypodense cells. The conditioned media from both eosinophil populations (hypo- and hyperdense) from donors 1, 2, and 3, significantly inhibited colony formation of PC-3 cells by greater than 90% ($P < 0.0001$) as shown in Figure 3B.

Eosinophil Cell Lines Inhibit Prostate Tumor Cell Growth

Our laboratory has developed eosinophil cell lines from peripheral blood eosinophils of allergic individuals because of the following reasons: (a) low numbers of eosinophils in peripheral blood (5%); (b) need of large volumes of blood from individual donors; and (c) inconvenience of multiple drawings of blood from the same donors. We have tested the functional activity of these cell lines against DU 145 and PC-3 tumor cell lines and the results are summarized in Figure 4A,B. Cell line GRC.014.22 (established from hypodense eosinophils) has no effect on DU 145 and PC-3 at E:T ratio of 1:1, minimal effect at 10:1 (20% and 10%, respectively) and a more variable effect at 25:1 (10% and 38%, respectively), as shown in Figure 4A. However, after treating these hypodense eosinophils with IL-5 (5 ng/ml), the growth inhibition of DU 145 increased by 12% at E:T ratio of 10:1 and by 30% at 1:1 ratio (Fig. 4A). When PC-3 tumor cells were used as targets for IL-5-treated hypodense eosinophils, the percent growth inhibition of tumor cells increased by 8% at E:T ratio of 25:1, by 30% at ratio of 10:1, and by 34% at the 1:1 ratio (Fig. 4A). Similar results were obtained from GRC.014.24 (hyperdense) cell line treated with and without IL-5 (Fig. 4B).

Conditioned Media From Eosinophil Cell Lines Inhibits DU 145 Colony Growth Formation

Eosinophil cell lines were cultured at 37°C for 24, 48, and 72 hr. Serial twofold dilutions were made of

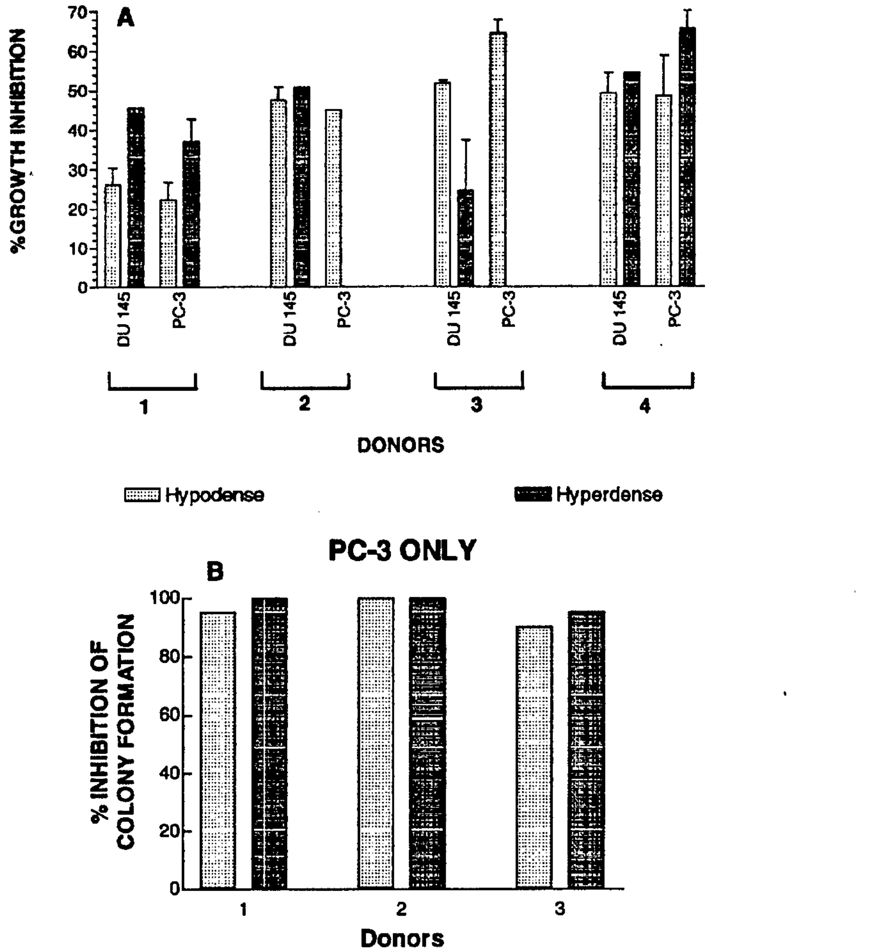

Fig. 3. Inhibition of prostate tumor cell growth by 24 hr eosinophil conditioned media. **A**: PC-3 and DU 145 tumor cells were seeded in replicates into 12-well plates at 1.5×10^5 cells/well and incubated overnight. The media was removed and replaced with 24 hr Peripheral Blood Eosinophil Conditioned Media from hypo- and hyperdense fractions of different donors. The plates were incubated for an additional 72 hr. After which the wells were rinsed 3 × with PBS, fixed with H&E, stained, and the percent growth inhibition was determined. **B**: Replicates of PC-3 tumor cells were seeded into 6-well plates at 10^2 cells per well and incubated overnight at 37°C. After 24 hr incubation, eosinophil conditioned media from various donors were added. The plates were incubated for an additional 10 days. The number of colonies was enumerated.

the captured supernatants (conditioned media) which were then examined for inhibition of DU 145 colony growth formation. The results are summarized in Figure 5A, B. As depicted in Figure 5A, GRC.014. 22 conditioned media, obtained from 24 hr incubation of this hypodense eosinophil cell line, inhibited growth of DU 145 colonies by 76% at ½ dilution, 42% at ¼ and 28% at the 1/8 dilution. The conditioned media at ½ dilution obtained from 48 and 72 hr incubations of this hypodense cell line gave comparable growth inhibition of DU 145 colony formation, 68% and 65%, respectively (Fig. 5A). Next, the conditioned media obtained from the FACS sorted CCR3$^+$ hypodense eosinophil cell subline, GRC.014.22S, were studied for their inhibitory effects on colony growth formation of DU 145 tumor cells. As shown in Figure 5A, the conditioned media obtained from all incubations (24, 48, and 72 hr) of GRC.014.22S dramatically abrogated colony growth formation of DU 145 tumor cells at all dilutions except the 1/8 dilution of the 24 hr incubation. This condi-

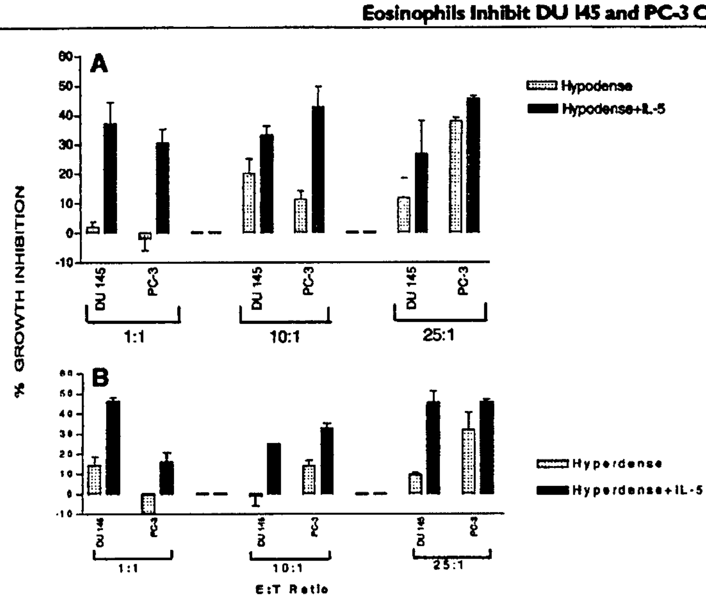

Fig. 4. Eosinophil cell lines inhibit prostate monolayer tumor cell growth. PC-3 and DU 145 tumor cells were seeded into 12-well plates at 1.5×10^5 cells/well and incubated overnight. Effector cells from the hypodense eosinophil cell line that were pretreated with IL-5 (1 ng/ml) for 24 hr were added in replicates to the seeded cells at various E:T ratios. The plates were incubated for an additional 72 hr, rinsed 3× with PBS, fixed, and then stained. The percentage of growth inhibition was determined.

tioned media inhibited colony growth formation of tumor cells by 70%. We also examined the conditioned media obtained from the culture of hyperdense GRC.014.24 eosinophil cell line, following incubation at 37°C for 24, 48, and 72 hr. The effects of these hyperdense conditioned media on the colony growth formation of DU 145 tumor cells are shown in Figure 5B. These conditioned media exerted greater inhibition of colony formation (Fig. 5B) than those of hypodense cell line, GRC.014.22 (Fig. 5A). In comparison, the conditioned media from the hyperdense cell line showed ≥90% inhibition of colony growth formation with the 1/2 dilution of media obtained from 48 and 72 hr incubations compared to 62–67% inhibition obtained with hypodense conditioned media at the same dilution. Furthermore, we examined the conditioned media obtained from FACS sorted $CCR3^+$ and $CCR3^+$, $VLA\text{-}4^+$ hyperdense eosinophil sublines, GRC.014.24S and GRC.014.24S.CD49d, respectively, on the colony growth formation of DU 145 tumor cells. The results are shown in Figure 5B. The conditioned media from GRC.014.24S demonstrated 100% inhibition of DU 145 colony growth formation with the 1/2 dilution of media obtained from 48 hr incubation and with all the dilutions of media obtained from 72 hr incubation, as shown in Figure 5B. The GRC.014.24S.CD49d conditioned media only gave 100% inhibition of DU 145 colony growth formation at the 1/2 dilution of media obtained from 48 and 72 hr incubations but exerted percent growth inhibitions that were superior to those of the hyperdense parental cell line GRC.014.24 with all the other dilutions at 48 and 72 hr incubations (Fig. 5B).

IL-4 and TNF-α Inhibit Tumor Cell Growth

Both IL-4 and TNF-α were detected in our eosinophil conditioned media (data not shown). We in turn studied the effects of commercial IL-4 and TNF-α on DU 145 and PC-3 monolayer cell growth. Overall, TNF-α was more effective in inhibiting both DU 145 and PC-3 growth. The percent growth inhibition ranged from 36% (10 ng/ml) to 100% (100 ng/ml) with the use of TNF-α, while the activity obtained with IL-4 was less than that of TNF-α. The maximal inhibition (42%) of DU 145 monolayer cell growth was obtained with 50 ng/ml of IL-4 (Fig. 6).

DISCUSSION

To the best of our knowledge, our data show for the first time that hypo- and hyperdense eosinophils from peripheral blood of allergic and asthmatic individuals inhibit growth of prostate tumor cell lines, DU 145 and PC-3 *in vitro*. These observations were made both with

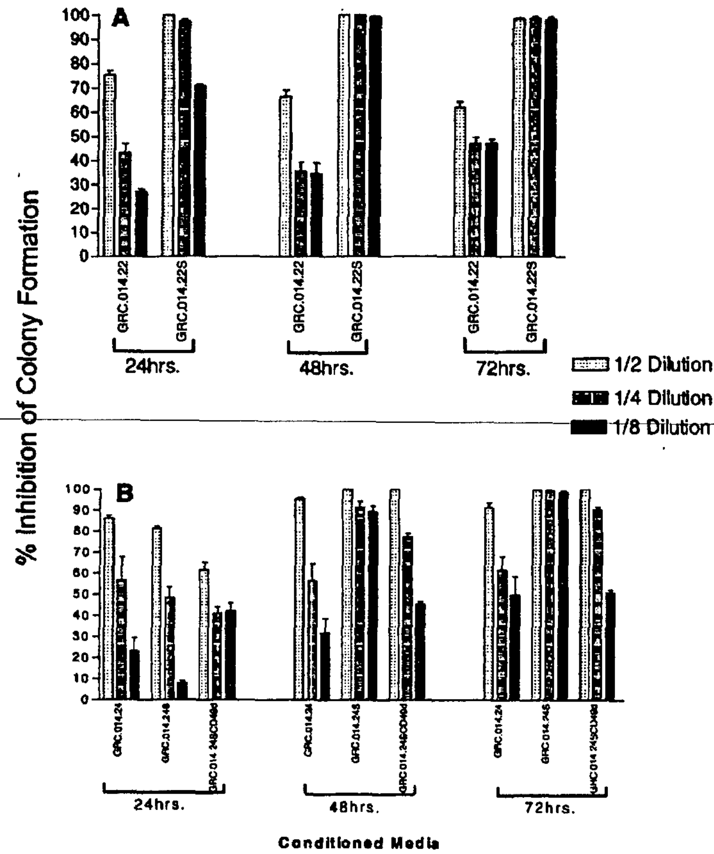

Fig. 5. Inhibition of DU 145 colony formation by eosinophil cell line conditioned media. DU 145 cells were seeded into 6-well plates in replicates at 10^2 cells/well and incubated overnight at 37°C. The wells were treated with various dilutions conditioned media obtained following incubations of eosinophil parental cell lines and their sublines for different 24, 48, and 72 hr. The number of colonies was counted for each dilution.

monolayer cell cultures and colony formation assays of these cell lines. Based on our studies, eosinophils in the hypo- and hyperdense fractions were effective in inhibiting growth of these prostate tumor cell lines. Hypodense eosinophils have been routinely found to be the most active and potent effectors of the two subpopulations of eosinophils in conditions such as asthma, allergy, and parasitic disease [40,41]. The fact that this was not seen in our studies could be due to the presence of hypodense eosinophils contaminating the hyperdense fraction. This is unlikely, however, because hypodense eosinophils, on metrizamide density gradients are located at a peak density between 1.3665 and 1.3680, while hyper- or normodense eosinophils are found between 1.3695 and 1.3710 [37–39]. These are reflected in the metrizamide percentages 21, 22 (hypodense), 23 and 24 (hyperdense), respectively. We selected fractions 22 and 24 for this study. If there were a mixture of the two subpopulations of cells, it would occur at fraction 23 (density 1.3695). Another possible explanation for this observed activity in the hyperdense subpopulation of eosinophils could simply be that these cells are removed from the peripheral blood of individuals who are in a state of hyperactivity (due either to an allergic or asthmatic reaction). Once out of the body, this state of activity is continuous for a short period of time without some kind of stimulus. Hence there are cells that have already released their granular contents (hypodense); those in the process of degranulating and still those at various stages of activation along this continuum. The hyperdense fractions may have been captured at a stage of readiness for

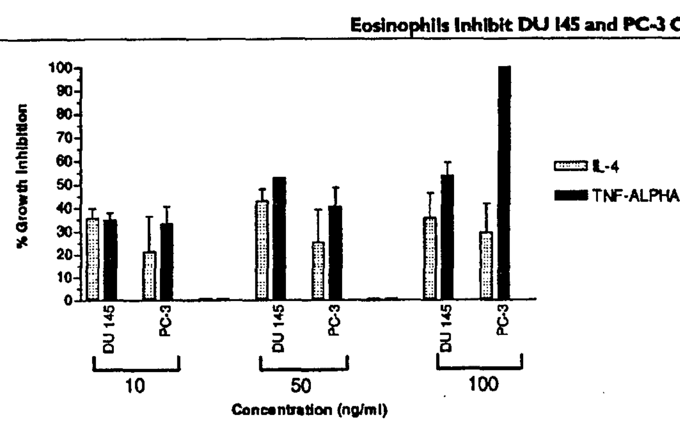

Fig. 6. Inhibition of prostate tumor cell growth by IL-4 and TNF-α. DU 145 and PC-3 cells were seeded into 12-well plates at 1.5×10^5 cells/well and incubated overnight. After 24 hr incubation, the cells were treated with IL-4 and TNF-α in replicates at various concentrations for an additional 72 hr. The wells were rinsed 3× with PBS, fixed, and then stained. The percentage of growth inhibition was determined.

degranulation to almost degranulating and hence upon capture *in vitro* could show potent activity upon degranulation. Piecemeal degranulation studies of peripheral blood eosinophils bear this hypothesis out [42].

Non-allergic eosinophils were less effective than allergic eosinophils as was expected since they were nonactivated and therefore contained far fewer granules, hence less toxicity. Although not shown here against prostate tumor cells, we have previously demonstrated that 24 hr supernatants from IL-5-treated non-allergic eosinophils inhibited breast tumor cells by as much as 60% (data not shown). One of the observations made from these studies is that eosinophils bound tightly to the tumor cells, moreso with DU 145 than PC-3, resulting in an apparent decrease in percent inhibition as the E:T ratio increased. Hence even in the presence of inhibition, bound eosinophils increased the opaqueness of the cell lawn and was measured by the densitometer, with measurements approaching that of the control. In the colony assay, bound eosinophils also gave the appearance of colonies and was therefore counted, thereby skewing the data somewhat. This skewing of data was more pronounced with the control eosinophils because the killing was not as dramatic as with the activated eosinophils.

There have been very few investigations on the role of eosinophils as anti-cancer effector cells in prostate cancer, but none have demonstrated effector growth inhibition by eosinophils as the present study has done. There have, on the other hand, been several studies on eosinophils in other cancers such as cervical, colon, lung, and gastric [23–29]. While tissue associated blood eosinophilia (TBE) has been associated with tumor spread and poor prognosis, tumor-associated tissue eosinophilia has been associated with a good prognosis [43–45]. The data from such observation remain inconclusive, however, as other investigators have not confirmed this [46–48].

Few, if any, studies have actually demonstrated eosinophilic infiltration of prostate tumors, however, the presence of eosinophil crystalloids in prostate tumor and hyperplastic tissue has been well documented [49–51]. This is a clear indication of eosinophil presence at some point in time. Moreover, Luna More et al. [52] have demonstrated an inverse relationship between the intensity of eosinophil crystalloids and Gleason grade.

In order to avoid the dilemma of low eosinophils numbers and still be able to have an available resource of eosinophils to carry out in depth studies of their biology, functional anti-cancer activity, gene expression profiles, etc., we have developed eosinophil cell lines from peripheral blood eosinophils of allergic and asthmatic individuals. In examining their functional activity against prostate tumor cell lines, our data show 30–38% killing by eosinophil cell lines GRC.014.22 and GRC.014.24 which is enhanced by as much as 24% by 1L-5. Colony formation assays revealed extreme potency of cultured supernatants from these parental cell lines as well as from their sublines, GRC.014.22S, GRC.014.24S, and GRC.014.24s.CD49d. Overall, the data clearly show that eosinophil and eosinophil sublines release products into the cultured medium which have very potent inhibitory effects on DU 145 and PC-3 cells.

Altogether these data, offer very intriguing insights into a potential role of eosinophils as anti-prostate cancer effectors. It is well-known that eosinophil chemokines as well as cytokines are able to recruit them into sights of inflammation [53,54] and moreover,

IL-5 and other cytokines or growth factors (e.g. IL-3, GM-CSF, IL-5) can activate eosinophils [55] to degranulate and unleash their toxic substances (e.g., MBP, ECP, EDN, EPO, O_2, LTC_4), all of which may have potential toxic effects on tumor cells [56]. These substances, along with the perforins and granzymes, support the hypothesis that the eosinophil is evolving as a potentially powerful, multi-functional (immunoregulatory, cytostatic, cytotoxic) cell in the immune arsenal against cancer, with several potential mechanisms of killing, e.g., antibody dependent cell cytotoxicity (ADCC), apoptotic induction, and cytolysis. Further studies have to be done to elucidate much of this mechanism as well as to develop strategies for enhancing eosinophilic presence and tumoricidal activity in prostate cancer.

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Dr. Paulette Furbent-Harris
Howard University
Cancer Center
2041 Georgia Ave NW
Washington, DC 20060
USA

April 6, 2004

Dear Dr. Furbent-Harris:

I am delighted to invite you to participate as a main speaker at the 7th **International Conference of Anticancer Research**, 25-30 October 2004, Corfu, Greece.

Your participation in all the scientific and social events will enhance the success of the conference.

This conference will comprise:

a. Regular sessions of oral and poster presentations.
b. Special sessions on topics of current importance.
c. The participation of ten scientific societies.
d. A session on the scope and establishment of the International Institute of Anticancer Research, Athens, Greece. This session will also include the description and discussion of several cooperative research projects and a detailed schedule of the activities of the International Conference of Anticancer Research.
e. Various social and cultural events.

Please note that according to our policy, we can only cover the registration fees of all invited scientists. This policy has enabled these conferences (a) to be independent and (b) to offer low accommodation fees for all participants. Also please note that Olympic Airlines, ANEK Lines and Minoan Lines offer significant discounts on their fares to Corfu for the conference. Detailed information about the conference is enclosed.

I am always grateful for your contributions and help.

I look forward to welcoming you to Corfu in October.

With my best regards,

Yours sincerely,

John G. Delinassios

John G. Delinassios
Director
IIAR

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Noisy-le-Grand, January 28, 2004

Dr.P.M.**FURBERT-HARRIS**
Howard University Cancer Center
2041 Georgia Av. NW #530
WASHINGTON DC 20060- USA

Dear Dr. Furbert-Harris

You have published recently a remarkable paper in my journal, the « Cellular and Molecular Biology® », 2003, 49(7):1009-1016, concerning the Suppressor Molecule E-Cadherin on tumor cells.

You have to know that we are highly interested to publish Themes which are of the greatest interest for our readers and scientists, particularly when there does not exist a recent book or survey on the matter.

That is the case with the Suppressor Molecule E-Cadherin which merits to be better known in all its implications in diverse tissues. Your contribution has therefore afforded new insights in its activity in the upregulation of the metastasis on prostate cancer. My proposal is to incite you to publish an entire issue of my journal devoted to the Suppressor Molecule E-Cadherin which plays a major role in many metabolisms and not only in cancer. I would be highly interested if you would explore all the aspects of this molecule of great interest.

That would need from you to solicit other authors to submit to you each one a paper on his researches on Suppressor Molecule E-Cadherin . In order to be able to publish one issue it is traditional that a "Guest Editor" of such an issue indicates to us about forty addresses, with complete data including e-mails so that we can write to these authors.

You send to us a solicitation letter, indicating your own data which we will integrate in a letterhead of our journal. We write all forty letters and sign them with your signature obtained by e-mail. We post them and you will receive the answers of the authors. At that moment you send us the list of all those having accepted. We take contact with the authors and give them all necessary indications how to present the paper, by requesting them to visit our site: cellmolbiol.com where they find them.

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If my proposal has an interest for you, please be so kind as to answer to me. I thank you very much in advance. Howard University is subscribing our journal since the beginning.

Yours very sincerely,

Professor Raymond J.WEGMANN, MD, DSci(State), Dhc (Japan & 14 others), MA
Member of the Academy of Science of Brazil
Former Dean of the Rutgers University Medical School, New Jersey

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Dr. Paulette Furbent-Harris
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April 7, 2004

Dear Dr. Furbent-Harris:

I am pleased to announce the organization of a special issue of **in vivo** on "New Anticancer Agents: *In Vitro* and *In Vivo* Evaluation". This issue will encompass all current research trends in the field of synthesis, evaluation and mechanism of action of new anticancer agents.

On behalf of the Editorial Board of **in vivo** I wish to invite you to contribute an original or review article for this special issue. Kindly follow the instructions to authors of **in vivo** and consider 31 October 2004 as a deadline for submission of your paper.

In 2003 **in vivo** has attained an exceptional popularity with over 500 subscriptions worldwide comprising all the main libraries of Universities, Hospitals and Industry. All submitted articles are expertly reviewed and rapidly published within 3-8 weeks from acceptance.

I also wish to invite you to the 7th International Conference of Anticancer Research, 25-30 October 2004, Corfu, Greece. Information and an invitation letter are enclosed.

With my best wishes,

Yours sincerely,

John G Delinassios

John G. Delinassios
Managing Editor